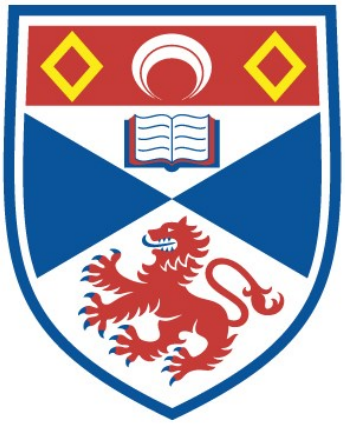
DISTRIBUTION, LOCALISATION AND FUNCTIONAL SIGNIFICANCE OF BIOLOGICALLY ACTIVE MONOAMINES IN GASTROPOD MOLLUSCS

Neville N. Osborne

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1970

ERRATA

In parts of this thesis

"Undenfriend" should read "Udenfriend"

"Kirschner" should read "Kirshner"

"Hökefelt" should read "Hökfelt"

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BY

N.N. OSBORNE B.Sc.

A thesis presented for the Degree of Doctor of Philosophy of the University of St. Andrews.

Wellcome Laboratories of Pharmacology
The Gatty Marine Laboratory,
The University,
St. Andrews.

May 1970.

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SUPERVISOR'S CERTIFICATE

I certify that Neville Osborne has fulfilled the conditions laid down under Ordinance No 16 of the University Court, St. Andrews, and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

[redacted]

DECLARATION

I declare that the work reported in this thesis is my own and has not previously been submitted for any other degree.

[redacted]

VITAE

I was educated at Sir John Cass College and Kings College London University where I graduated in Zoology in 1966. The work described in this thesis was carried out between February 1967 and May 1970.

ACKNOWLEDGEMENTS

I should like to express my sincere thanks first and foremost to Dr. G.A. Cottrell for giving me the opportunity to work under his guidance, for his supervision, encouragement and constructive criticisms throughout my work; to Professor M.S. Laverack for his assistance in many ways; to Mr. J. Brown for his willingness and help in providing materials and to Mrs. J. Thompson for typing the manuscript. My gratitude is also due to my fiancée for reading and correcting the manuscript in proof, and to my family, especially my late father, for constant encouragement. Finally, I am indebted to the Wellcome Trust for providing the money which made this study possible, and to the pulmonate molluscs for having giant neurons.

INTRODUCTION AND REVIEW OF LITERATURE

CHEMICAL TRANSMISSION AT THE SYNAPSE

Although nerve cells are isolated morphologically, they are nevertheless interconnected physiologically through junctions known as synapses. These are specialised regions where the processes of a neuron (presynaptic regions) come into close contact with effector cells (postsynaptic regions). A major problem in synaptic transmission is to ascertain the method whereby a nerve impulse passes from one neuron to another, across a synaptic cleft. It is now thought that chemical transmission at the synapse is through the release of a substance from within the presynaptic terminal onto the post-synaptic membrane. The transmitter affects receptor sites on the latter with a resultant selective change in the permeability properties of the membrane.

Du Bois Reymond (1877) was the first to suggest that synaptic transmission could be induced by chemical agents. It was not until 1904, however, that Elliot provided evidence for this view. He noted the resemblance between sympathetic stimulation and the

effects of administered adrenaline (AD), and concluded that AD might be the chemical stimulant liberated each time the impulse arrives at the periphery. But we know now that noradrenaline (NA) and not AD is the real sympathetic transmitter (see De Robertis 1964). A year later, Langley (1905), proposed that cells possess both excitatory and inhibitory substances and that the response to the chemical agents depends upon which substance is present. He spoke of "the receptive substance" or special cell constituent which might initiate the physiological response. Dixon (1907) proposed that the vagal nerve on stimulation released a muscarine-like substance which combined with the receptor to produce the response. This work was extended by Dale (1914), who drew attention to the similarity between the effects of administered acetylcholine (ACh), and stimulation of the parasympathetic system. However, it was not until 1921, that Loewi provided convincing evidence for chemical mediation of nerve impulses by the peripheral release of specific chemical substances. He arranged two frog hearts in series so that the perfusion fluid from one passed into the second. Vagal stimulation of the

first heart produced a slowing down of both hearts, and it became apparent that a vagomimetic substance ("Vagustoff") had been liberated which produced effects identical to those from chemical stimulation. The accumulation of data suggested that transmission at the synapse could be mediated by the action of chemical substances.

Experimental evidence for the release of transmitter substances in multimolecular packets, or quanta, from presynaptic regions was provided by Fatt and Katz (1952). A quantum may correspond to several thousand ACh molecules simultaneously released at a definite locus of the synapse. This quantal release was recorded from the myoneural region by minute transient fluctuations in voltage; these are called miniature end-plate potentials. Later, Katz (1959) suggested that under the action of a nerve impulse there would be a synchronous discharge of a certain number of quantal units which give rise to the much larger synaptic potential.

In 1954, De Robertis and Bennet made the important discovery that certain synapses contain vesicles about 30 mm in diameter. These they termed

'synaptic vesicles' and interpreted them as storage sites for the transmitters. Since then, fractionation studies by a number of workers have shown transmitter substances to be associated with such particles (see Whittaker 1966 and page 28). By electrically stimulating a peripheral synapse, De Robertis and Vas Ferreira (1957) were able to demonstrate a significant change in the number of vesicles, and this was related to the frequency of the stimulus. Del Castillo and Katz (1956) had previously made the correlation between the presence of synaptic vesicles and the quantal release which occurred in synaptic transmission.

In 1959, Gray recognised that on the basis of morphology alone, the synapses in the cerebral cortex fall into two main categories. Anderson and Eccles (1965) concluded furthermore that the one category is excitatory and the other inhibitory. Attempts to correlate morphology with function however, can be misleading (see Bodian 1966), for it is now generally accepted that many synapses exhibit structural features intermediate between the two categories of synapses originally recognised by Gray.

There is nevertheless growing support for the postulated identification of synaptic function by the shape and size of synaptic vesicles. Uchizono (1967), has published a detailed account of his electron microscopic investigations on the cerebellum in which he showed a correlation between the physiological identification of synapses as excitatory or inhibitory, and the spheroidal or ellipsoidal shape of the vesicles.

Recent experimental investigations, especially on the nervous systems of invertebrates, show that the idea that all neurons have a specific effect, namely excitatory or inhibitory, is not necessarily true. Although it seems a general rule that a neuron never produces more than one transmitter, the responses it elicits on the post synaptic cell can be different. For example, in the gastropod molluscs there are two classes of cells, the D- and H- cells, which react to acetylcholine (ACh), with depolarisation and hyperpolarisation respectively. Moreover, it has been shown that in Aplysia (Tauc and Gerschenfeld 1961; Strumwasser 1962; and Kandel, Frazier and Coggeshall 1967) a single interneuron which makes direct synapses

with a D- and H- cell, can excite the D- cell and inhibit the H- cell through the same transmitter, ACh. Examples like this show that the nature of the post-synaptic membrane is important in determining the effect of the released transmitter; i.e. a single nerve cell can excite one neuron and inhibit another. It is for these reasons that the classification of synapses as inhibitory or excitatory should be avoided.

It has become evident that the important feature of a chemical synapse is the nature of both the transmitter substance and the post-synaptic membrane. Cottrell and Laverack (1968) have emphasised that there is no reason to believe that a transmitter substance cannot have its effect by acting intracellularly as do some hormones. The best established transmitter is ACh, which is thought to be the excitatory transmitter on the vertebrate somatic muscle end-plate and on some molluscan neurons, and also the inhibitory transmitter on vertebrate cardiac muscle. Other substances with strong claims to be considered as transmitter are GABA, glutamate and the monoamines.

This thesis is concerned with providing data to emphasise the importance of the monoamines, particularly 5-hydroxytryptamine (5-HT), in the neurophysiology of gastropod molluscs.

HISTORICAL INTRODUCTION TO BIOLOGICALLY ACTIVE MONOAMINES

1. CATECHOLAMINES

The study of catecholamines has a history that goes back over a period of more than 100 years (see Blaschko 1957). Their very specific chemical properties were first noted by Vulpian (1857), and a few years later Henle (1865) described the reaction of the adrenal medulla with potassium dichromate a reaction which gave rise to the name "chromaffin" (Kohn 1903) or "phaeochrome" (Poll 1906) cell. Following the isolation of adrenaline (AD) from the adrenal medulla (Takamine 1901), Balfour (see Boyd 1960) traced the origin of the chromaffin tissue (and the sympathetic ganglion cells) to the neural crest and thus laid the foundation of our ideas on the relationship between nerve cells and catecholamine

secreting cells. His ideas found independent support in the observations of Poll on chromaffin ganglion cells in Annelids (Poll and Sommer 1903). It was at this time that the concept of chemical transmission at the synapse was first proposed by Elliott (1904). He suggested that AD might act at the junction of sympathetic nerves and the effector organs, and that AD might be released at sympathetic nerve endings. Because of the similarities between the effects of sympathetic nerve stimulation and those of AD it was assumed that the transmitter liberated at sympathetic nerve endings was AD (Loewi 1921). This view was to remain generally accepted until 1945 (see Dale 1960), when it was shown that NA was the sympathetic transmitter in mammals.

As early as 1939, Blaschko proposed that in the formulation of AD from tyrosine four steps were necessary (see Fig 1). The first direct evidence for this hypothesis came from studies of the synthesis of catecholamines in the adrenal medulla. It is now well established that adrenal medulla tissue, both in vivo and in vitro, is able to convert radioactive labelled

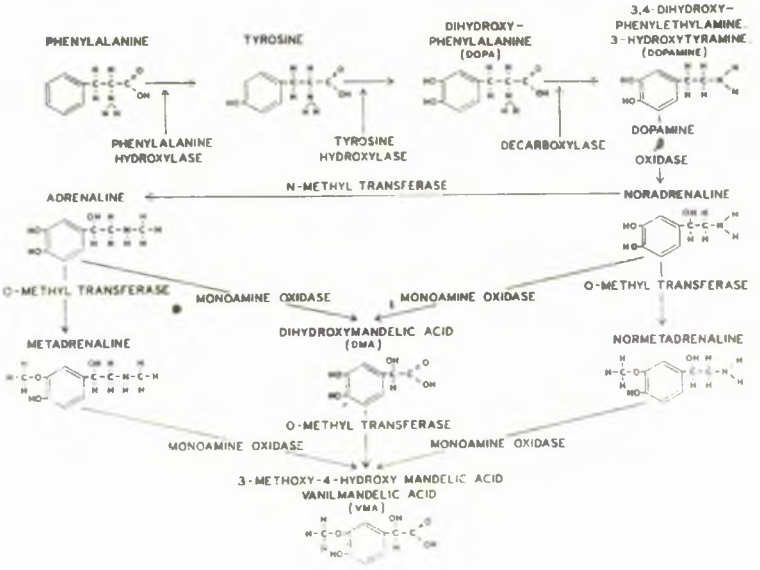
tyrosine or DOPA into DA, NA and AD (Dewis, Blaschko and Welch 1953; Kirshner and Goodall 1956; and Udenfriend and Wyngaarden 1956). With the advent of chromatographic techniques for the separation of the catecholamines from their precursors and metabolites, and with the availability of radioactive labelled precursors of high specific activity, the biosynthesis of the catecholamines in tissues other than adrenal medulla and in particular in nervous tissue was demonstrated (e.g. Goodall and Kirshner 1958; Masuoka, Schott and Petriello 1963; and Spector, Sjoerdsma, Zaltman-Nirenberg, Levitt and Udenfriend 1963).

As knowledge of the biochemistry, pharmacology and distribution of the catecholamines increased, it became clear that besides being precursors of AD, the primary catecholamines (DA and NA) have potent physiological actions of their own. It now seems probable that DA (see Bertler and Rosengren 1966; and Hornykiewicz 1966) and NA (see Euler 1956; Glowinski and Baldessarini 1966; and Iversen 1967) could have independent roles in different situations

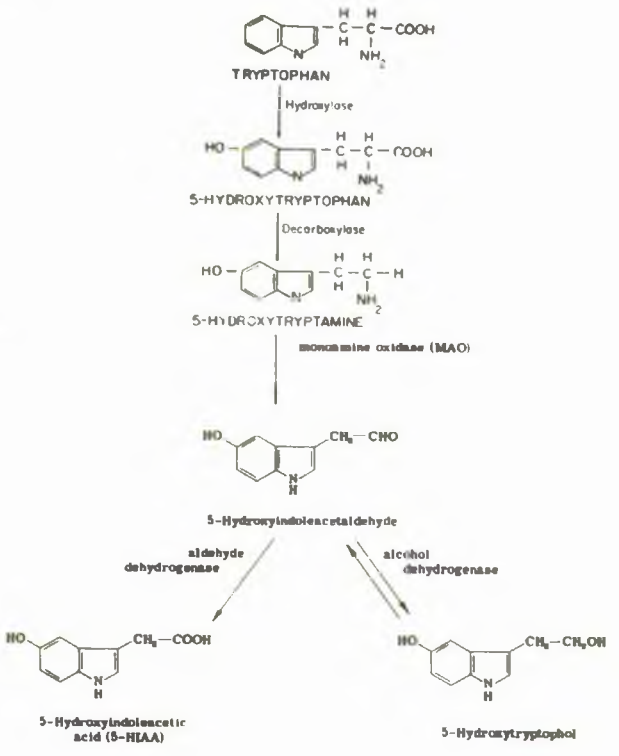
Fig 1. Formation of catecholamines and their metabolites from phenylalanine.

Fig 2. Formation of 5-hydroxytryptamine and metabolites from tryptophan.

1


PHENYLALANINE
TYROSINE
DIHYDROXY-PHENYLALANINE (DOPA)
3,4-DIHYDROXY-PHENYLETHYLAMINE 3-HYDROXYTYRAMINE (DOPAMINE)
PHENYLALANINE HYDROXYLASE
TYROSINE HYDROXYLASE
DECARBOXYLASE
DOPAMINE
OXIDASE
N-METHYL TRANSFERASE
ADRENALINE
NORADRENALINE
O-METHYL TRANSFERASE
MONOAMINE OXIDASE
MONOAMINE OXIDASE
O-METHYL TRANSFERASE
METADRENALINE
DIHYDROXYMANDELIC ACID (DMA)
NORMETADRENALINE
O-METHYL TRANSFERASE
MONOAMINE OXIDASE
MONOAMINE OXIDASE
3-METHOXY-4-HYDROXY MANDELIC ACID VANILMANDELIC ACID (VMA)

2


TRYPTOPHAN
Hydroxylase
5-HYDROXYTRYPTOPHAN
Decarboxylase
5-HYDROXYTRYPTAMINE
monoamine oxidase (MAO)
5-Hydroxyindoleacetaldehyde
aldehyde dehydrogenase
alcohol dehydrogenase
5-Hydroxyindoleacetic acid (5-HIAA)
5-Hydroxytryptophol

and also be precursors in the formation of AD, which has functional effects of its own (see Marley 1966).

2. 5-Hydroxytryptamine (5-HT)

Erspamer and co-workers (see Erspamer 1954) obtained from rabbit gastrointestinal mucosa and salivary glands of octopods, acetone extracts which could stimulate the intestinal muscle. No substance known at that time could account for this effect, so they concluded that they had identified a new substance and called the active factor of the extracts enteramine. Later, Rapport, Green and Page (1948) used acetone to isolate from blood serum a substance which they called serotonin, and which caused the contraction of both isolated blood vessels and isolated stomach strips. Subsequently, both enteramine (Erspamer and Asero 1952) and serotonin (Rapport 1949) were shown to be identical to 5-HT. With the detection of the amine in the central nervous system (Amin, Crawford and Gaddum 1954) and the discovery that several compounds structurally related to 5-HT block its effect on smooth muscle

(Gaddum 1953) and can influence mental activity (Stoll 1947; Gaddum 1953; and Woolley and Shaw 1954), it was thought that 5-HT might function as a chemical transmitter in the brain. Work on the pharmacology, biochemistry and distribution of the indole alkylamine expanded rapidly (see Erspamer 1966) when it was shown by Pletscher, Shore and Brodie (1956) that certain tranquillizing drugs reduce the amounts of newly discovered 5-HT in cerebral tissue. We now know that tryptophan is the dietary precursor of 5-HT, and that 5-hydroxy-tryptophol and 5-hydroxyindoleacetic acid are the major end products. (see fig 2).

OCCURRENCE AND REGIONAL DISTRIBUTION OF MONOAMINES

1. INVERTEBRATES

An extensive survey of catecholamines in the invertebrates was undertaken in 1954 by Östlund using paper chromatography and biological assay methods. He indentified DA, NA and AD in larvae of many insects, and established a correlation between amine concentration and the development stage. Another investigation carried out by Frontali and Norberg (1966) on the brain of Periplaneta americana showed 5-HT present, in addition to catecholamines. In the crustacea, it has been estimated that 7μg/g DA (Kerkut, Sedden and Walker 1966; and Cottrell 1967a) and 0.1μg/g 5-HT (Welsh and Moorhead 1960) are in the nervous system of Carcinus maenus.

Cottrell (1967a) investigated the presence of catecholamines in the echinoderms, using paper chromatography. He demonstrated that the radial nerves of the starfish and sea urchin contained 5-16 μg/g DA and 1-7 μg/g NA respectively. There is no evidence for the occurrence of 5-HT in the echinoderms (see Pentreath 1970).

Sweeney (1963) carried out an investigation into the catecholamine content of pooled ganglia of molluscs, employing the techniques of chromatography and spectrophotofluorometry. The only catecholamine detected was DA, the concentration of which varied in different species with a maximum concentration of 261 µg/g in Mercenaria mercenaria. Cardot (1963), working on Helix pomatia, estimated colorimetrically that brain extracts contained 2-4 µg/g DA. This is in agreement with the results of Dahl, Falck, Lindqvist and Macklenburg (1962) who used spectrophotofluorometry and found that the cerebral ganglia of Helix pomatia contained 7.25 µg/g DA and 3.77 µg/g 5-HT. The 5-HT content of the circumoesophageal ganglia of Helix aspersa has been calculated by Markat and Cottrell (1963) to be 0.3-4 µg/g.

Using paper chromatography, Cottrell (1967a) showed that the brain of Eledone cirrhosa (cephalopod) contained 16-26 µg/g DA and 4-10 µg/g NA and that the pooled ganglia of Sepiola solida (lamelli branch) had 80-100 µg/g DA and 10-12 µg/g NA. The only previous author to detect NA in the molluscs was Puppis (1964)

who found traces of NA and also AD in Anadonta cygnea (lamelli branch). Recently Sweeney (1968) demonstrated fluorometrically the occurrence of 5-HT, DA and NA in the Sphaerium sulcatum. There is no evidence for the occurrence of either NA or AD in the gastropods.

A survey for the distribution of 5-HT in the invertebrates has been carried out by Welsh and Moorhead (1960) and Erspamer (1966). The ganglia of lamellibranch molluscs contain the highest levels of 5-HT found so far in any nervous tissue (8.60 µg/g). Ganglia of gastropod (1.2-11 µg/g) and cephalopod (0.7-4 µg/g) molluscs contain less 5-HT than do the lamellibranchs. The amine besides being present in nervous tissue also occurrs in non-nervous tissues. The toxin producing salivary glands of Octopus vulgaris contain 300-500 µg/g 5-HT (Erspamer 1948). Particularly large amounts have since been detected in the venum apparatus of other molluscs, scorpions, insects and coelenterates. 5-HT has also been observed to occur in high quantities in the green glands of crabs (Welsh and Moorhead 1960) and the spermatophore

sac of octopus. (Munn 1963).

In his survey in 1954, Östlund showed the presence of NA and AD in the nerve cord of Lumbricus terrestris (Annelida). Von Euler (1961) estimated spectrophotofluorometrically the concentration of catecholamines in this animal and found 0.003 µg/g AD and 0.015 µg/g NA. Recently Rude (1969) using thin layer chromatography, proved that extracts of ventral nerve cords from earthworms contain large amounts of DA, traces of NA, but no AD. Employing spectrophotofluorometric methods, Welsh and Moorhead (1960) showed the occurrence of 5-HT in a variety of annelids; it was estimated that the earthworm contained 7-10 µg/g 5-HT.

Biogenic amines also occur in the protozoa. Using spectrophotofluorometric methods Janakidevi, Dewey and Kidder (1966) found 0.11-0.13 µg/g 5-HT and 0.1-0.2 µg/g NA in the flagellate Crithidia fasciculata and 0.25-0.35 µg/g NA and 0.13-0.15 µg/g AD in the ciliate Tetrahymena pyriformis.

2. VERTEBRATES

The regional distribution of 5-HT, NA and DA in a variety of vertebrate tissue has been reviewed and

summarised by Brodie, Bogdanski and Bonomi (1964). 5-HT is present in all the vertebrate classes. Its concentration in the Reptilia and Amphibia is generally high in relation to the mammals. In the brain of the toad Bufo americanus, the level is extraordinarly high, averaging 9 µg/g. However in the brain of two fish species the concentration of 5-HT is even lower than the mammals. The gastro-intestinal tracts of mammals, reptiles and amphibians contain on the whole considerably more 5-HT than those of the two fish species studied. The catecholamines in amphibian brain consist largely of AD, whereas it is mainly NA in the brain of vertebrates above and below Amphibia in the phylogenetic scale. AD is also predominant in peripheral organs of frogs and toads, though there are appreciable amounts of NA in the stomach of these animals. In the peripheral organs of salamanders however, the main catecholamine appears to be NA.

A wide variety of mammalian brains have been studied by Bertler and Rosengren (1959), and in all cases the distribution pattern of DA and NA was different. The distribution of DA in the brain of some mammalian

species has been summarised by Hornykiewicz (1966). A survey carried out on the spinal cord by McGeer and McGeer (1962) showed that DA content decreased with increase in size of the animal, for example in the rat the DA content is 1 µg/g and in the ox it was 0.2 µg/g. Haggendal and Malmfors (1965) found that the retina of the rabbit contained higher amounts of DA than NA whilst the inverse was the case in the choroid plexus. Other peripheral tissue, heart, spleen, kidney and liver of a wide variety of mammalian species and the turtle have been shown to contain DA (Anton and Sayre 1964).

The general distribution of biogenic amines in the vertebrates indicate that DA, NA, AD and 5-HT may have physiological functions of their own in the central and peripheral nervous systems. In the brain, physiologically older portions contain higher concentrations of amines than the newer parts; this distribution indicates that amines are concerned with autonomic sympathetic functions and with the emotional aspects of behaviour.

3. PLANTS

Erspamer (1966) surveyed the distribution of 5-HT and catecholamines in plants. The nut Juglans regia contains 340 µg/g 5-HT (Kirberger and Braun 1961) and as much as 700 µg/g DA and 122 µg/g NA is present in the outer peel of a ripe banana (Waalkes, Sjoerdsma, Creveling, Weissbach and Undenfriend 1958). The significance of monoamines in plants is not understood. It may be that in some cases they act as growth factors or as precursors of auxins and in other circumstances they intervene in the synthesis of pigments. It is highly possible that they are merely metabolic end products with no particular significance.

CELLULAR LOCALISATION OF MONOAMINES IN THE INVERTEBRATE

The histochemical fluorescence method of Falck and Hillarp makes it possible to demonstrate certain biogenic amines (DA, NA, and 5-HT) and related substances at the cellular level (for references see Corrodi and Jonsson, 1967). The method is extremely sensitive and specific for the localisation of catechol-and indoleamines, and must be judged by a number of criteria recommended (see Falck and Owman, 1965) to distinguish between specific and non-specific fluorescence in formaldehyde-treated samples. When monoamines in freeze-dried tissues are exposed to formaldehyde vapour at 80°C, highly fluorescent green fluorophores are formed from catecholamines and yellow fluorophores from 5-HT (see page 88). During recent years much information on this subject has been published dealing with the vertebrates, especially the mammals (for references see Norberg and Hamberger, 1964; Falck and Owman, 1965; and Anden, Carlsson and Haggendal, 1969). There are by comparison few investigations of invertebrate groups which will be reviewed.

1. MOLLUSCS

The molluscs were the first to be investigated among the invertebrates; Dahl and co-workers, (Dahl, Falck, Lindqvist and Mecklenburg, 1962; Dahl 1963; and Dahl, Falck, Mecklenburg, Myhrberg and Rosengren, 1966) described the distribution of monoamines in a number of molluscs, particularly Anodonta piscinalis (lamellibranch) and Helix pomatia (gastropod). The cerebral ganglion of A. piscinalis revealed a large number of yellow fluorescent and green fluorescent cells with short processes. The centre of the ganglion contained mainly green varicose fibres, and a few yellow varicosities arranged in rows. In the circum-oesophageal ganglionic mass of H. pomatia a number of cell bodies emitted either a green or yellow light. The majority of neurons did not fluoresce at all. These cells that contained monoamines varied considerably in size and shape, only moderate fluorescence was associated with the medium or very large perikarya, compared with the intense fluorescence in small cells.

Dahl and co-workers noted a number of similarities between molluscan and vertebrate monoamine containing neurons:

(a) The morphology of the neurons is the same in that the axons consist of a smooth preterminal part and a varicose terminal part. Exceptions to this general morphology exist in both vertebrates and invertebrates.

(b) The intraneural distribution of monoamines is similar; the terminal varicose part always shows a high fluorescence intensity.

(c) As in the vertebrates, a depletion of monoamines can be produced by means of treatment with reserpine.

(d) Fluorescent varicose fibres have been seen to superimpose on non-fluorescent cell bodies and this occurs in a similar manner to the vertebrate systems.

The general distribution of monoamine fluorescence in the lamellibranch is as follows. A number of green and yellow neurons are present in the pedal, visceral and cerebral ganglia. The neuropiles of these ganglia are densely packed with green and yellow fluorescing fibres (Dahl, 1963; Dahl, Falck, Lindqvist, Mecklenburg, Myhrberg and Rosengren, 1966; Zs-Nagy 1968; Sweeney 1965; and Japha and Wachtel, 1969). In Sphaerum sulcatum, Sweeney (1968) showed

that although the majority of neurons did not fluoresce at all, the pedal ganglion contains only yellow 5-HT cells, the visceral only green catecholamine types and the cerebral ganglia both types of cells. Catecholamine containing neurons have also been reported in foot and mouth palps of Anodonta piscinalis (Dahl, 1963; and Dahl, Falck, Macklenburg and Myhrberg, 1963a) and the peripheral structures of Mya arenaria (Sweeney 1965). Green cells have also been shown to occur in the foot, siphon, statocysts, anterior mantle edge, gill filaments and three accessory ganglia (buccal, genital and posterior pallial) of Sphaerum sulcatum (Sweeney, 1968). Sweeney (1968) suggests that catecholamine cells in the ganglia of lamellibranchs are associative neurons, whilst in peripheral tissue their function is sensory. Za-Nagy (1967b) maintained that 5-HT in Anodonta cygnea is not a transmitter agent but instead is involved in the regulation of such processes as spontaneous activity. Dahl, Falck, Macklenburg, Myhrberg and Rosengren (1966); Sweeney (1968); and Japha and Wachtel (1969) interpreted the yellow fluorescing cells as indicating a transmitter role for 5-HT.

The detailed distribution of monoamines in the central nervous system has been studied in the gastropods. Sedden, Walker and Kerkut (1968) mapped out the distribution of monoamine-containing neurons in the circumoesophageal ganglionic mass in Helix aspersa, with special emphasis on the location of large and giant cells. Sakharov and Zs-Nagy (1968) showed the occurrence of a similar distribution of yellow and green cells in the cerebral ganglia of Lymnea stagnalis. Although the majority of fluorescent perikarya appears either yellow or green, Kerkut, Sedden and Walker (1967) noted that some cells at the junction of visceral and right parital ganglia in Helix aspersa were yellow-green in colouration and evidence was presented which indicated that these cells contain DA and 5-HT. It therefore appears as if there are at least four types of cells in the gastropod central nervous system: (1) those that contain no monoamines, (2) catecholamine-containing cells, (3) 5-HT-containing cells, (4) cells containing both catecholamines and 5-HT.

The distribution of monoamines in other tissues of gastropods has not been studied in detail. Rózsa and Zs.-Nagy (1967) showed the presence of green nervous elements in the heart of Lymnaea stagnalis, and suggested that 5-HT known to occur in heart extracts was localised in muscle cells. The tentacles of Buccinum undatum contain green subepithelium nerve cells which send fibres towards the epithelium (Dahl, Falck, Mecklenburg and Myrhberg 1963a). Recently Foh and Begunch (1968) showed the occurrence of monoamine-containing nerve fibres in the penis retractor muscle of Helix pomatia. Monoamine nerve cells have also been observed to occur in the cephalic tentacles of Littorina littoria (Storch and Welsch 1970) and the foot of Helix pomatia (Rodgers, 1969).

2. OTHER INVERTEBRATE PHYLIA

There has been extensive research into the localisation of monoamines in the annelids e.g. Oligochaeta (Rude 1966; Kerkut, Sedden and Walker 1967; and Myrhberg 1967) Polychaeta (Clark 1966) and Hirudinea (Kerkut, Sedden and Walker, 1967; Maraden and Kerkut, 1969; and Rude 1969). The general picture which emerges is that the central nervous system contains

many yellow fluorescent neurons but only a few green cell bodies, with an abundance of both fibre types in the neuropile. In the peripheral nervous system catecholamine containing neurons are numerous, whilst 5-HT cells are never observed. Clark (1966) and Myhrberg (1967) suggest that some of the 5-HT bearing axons are motor in function. Rude (1966) considers that at least some of the yellow fluorescent cells in the ventral nerve cord of Lumbricus terrestis are interneurons. Many of the catecholamine containing neurons, especially in the peripheral nervous system, are thought to be sensory in function (Clark, 1966; Rude 1966; Myhrberg 1967; and Rude 1969).

In the crayfish Astacus astacus, numerous monoamines, especially catecholamine neurons, occur in the protocerebrum, external medulla, internal medulla and ventral nerve cord. Varicose monoaminergic fibres are especially prominent in those parts of the brain which are held to be associative in function (Elofsson, Kauri, Neilson and Stromberg 1966). Catecholamines have also been described as occurring in nerve fibres innervating the gut of Astacus astacus (Elofsson, Kauri, Neilson and Stromberg 1968) and in the stomatogastric ganglion of Homorus vulgaris (Osborne and Dando 1970).

The distribution of monoamines in the cerebral and suboesophageal ganglia of Trichoptera (Insecta) has been studied by Klemm (1968). Catecholamine structures appeared densely packed in the external medulla, internal medulla, ventral corpus, central corpus, α and β lobes, and in the suboesophageal ganglion. In the deutocerebrum, green fluorescence was less abundant and yellow fluorescence was never observed. This was in agreement with the findings of Frontali and Norberg (1966) and Frontali (1968) of a similar system of catecholamine distribution in the cockroach.

In the Nemertina, only green specific fluorescence could be found (Reutter 1969). The brain contains a few catecholamine cells which supply mainly the three peripheral nets of nerve fibres, situated in the frontal organs, in the proboscis and in the mouth fore gut region. Reutter (1969) suggests that the function of catecholamines is both sensory and motor.

The localisation of monoamines in four

species of Echinodermata has been studied by Cobb (1969) and Cottrell and Pentreath (1970). Fluorophores showing the presence of 5-HT were never observed. Green fluorescent neurons were confined to the ectoneural nervous system. It is though that these catecholamine neurons represent a class of interneurons, the function of which is not clear, but may have a role in the activity of the tube feet (Cottrell and Pentreath 1970).

Welsh (1968) studied the distribution of 5-HT in the planarian Phagocata oregonensis. Whole planarians, freeze-dried and then exposed to formaldehyde gas, showed well defined cerebral ganglia in which two to four pairs of yellow fluorescing cells could be seen when viewed under ultra violet light. In a later survey Welsh and Williams (1970) found neurons containing either 5-HT or primary cate cholamines, not only in the cerebral ganglia but also in the ventral nerve cord, the peripheral plexus and the pharynx. The fluorescing components of the peripheral plexus were mostly catecholamine-containing in dorsal regions, and 5-HT-containing in ventral regions.

Sea anemones have been shown to contain only green neurons in their tentacles (Dahl, Falck, Mecklenburg and Myhrberg 1963b). This system consists of bipolar cell bodies in the ectodermal epithelium and a feltwork of fluorescent fibres with numerous varicosities forming the subepithelium network. The authors state that the cells are sensory in function.

A different histochemical method (used to localise catecholamines in the adrenal medulla) was employed by Wood and Lentz (1964) to show the presence of 5-HT and catecholamines in ganglion cells, sensory cells and their neurites of Hydra. The same method was used by Lentz (1966) to reveal catecholamines in spindal shaped bipolar and multipolar cells in the mesenchyme of the sponge Sycon alatum.

SUBCELLULAR DISTRIBUTION OF MONOAMINES

1. VERTEBRATES

Strong evidence has been put forward to show that small granular vesicles (diameter about 40-100nm) are storage sites of DA, NA and 5-HT in peripheral and central nervous systems, and that the presence of a dense

core is related to the localisation of the monoamine. I shall summarise this evidence:

(a) As early as 1956 von Euler and Hillarp presented evidence that NA in peripheral adrenergic neurons is present in high concentrations in subcellular particles. This was collaborated by further subcellular fractionation studies, revealing NA (e.g. Euler and Swanbeck 1964; Potter 1966; and Sosa-Lucaro, de la Iglesia, Lumb, Berger and Bencome 1969), 5-HT (e.g. Whittaker 1959; Michaelson and Whittaker 1962; Zieher and De Robertis 1963; and Wesemann 1969) and DA (e.g. Weil-Malherbe and Bone 1959; Laverty, Michaelson, Sharman and Whittaker 1963) to be associated with vesicles.

(b) The histochemical fluorescence method made it possible to perform electron microscopical studies on areas known to contain high concentrations of monoamines. For example in the brain, the caudate nucleus (DA), periventricular region of the hypothalamus (NA) and the supra chiasmatic nucleus of the hypothalamus (5-HT) have been examined. Small granular vesicles were present in all three areas (see Fuxe, Hökefelt and Nilsson 1965).

Subsequent electron microscopical studies disclosed the occurrence of granular vesicles in certain peripheral neurons (e.g. De Robertis and Pellegrino de Iraldi 1961; Taxi 1961; Richardson 1962, 1964 and Hökfelt 1968), where monoamines were known to occur.

(c) Combined use of biochemical and histochemical studies (Vogt 1954; Malmfors 1965; and Zapata, Hess, Bliss and Eyzaguirre 1969) have shown that a general correlation exists between the localization of monoamines and the occurrence of small granular vesicles.

(d) The granular vesicles are concentrated, especially in certain parts of the terminal axons - called 'nerve endings' or 'boutons' (Richardson 1964) - which are similar in size and shape as demonstrated by fluorescence microscopy.

(e) Autoradiographic studies (e.g. Wolfe, Axelrod, Potter and Richardson 1962; Lenn 1966 and Aghajanian and Bloom 1967), reveal activity in nerve endings containing granular vesicles, and support both the fact that granular vesicles are storage sites of

monoamines, and that nerve endings containing large amounts of vesicles correspond to strongly fluorescent varicosities as seen in the fluorescence microscope.

(f) Denervation (e.g. van Orden, Langer and Trendelenburg 1967) and ligature (e.g. Kapeller and Mayor 1967, and Geffen and Ostberg 1969) experiments when studied biochemically, histochemically and electron microscopically showed a correlation between dense core vesicles and monoamine concentration.

(g) The dense core of the small granular vesicles can be made to disappear by treatment with certain drugs known to deplete monoamine stores (e.g. Fuxe, Hökefelt and Nilsson 1965; Bloom and Barrnett 1966 and Pellegrino de Iraldi and Guedet 1969). Furthermore, after electrical stimulation in combination with synthesis inhibition, no small granular vesicles can be seen but only agranular ones (e.g. Hökefelt 1967).

(h) After depletion with certain drugs, or stimulation in combination with synthesis inhibition, the dense core reappears after administration of exogenous amines <u>in vitro</u> (e.g. van Orden, Bensch and Giarman 1967, and Snipes, Thoenen and Tranzer 1968).

(j) In vivo and modal experiments have shown that the reaction between amines and certain fixatives (e.g. glutaraldehyde, osmium tetroxide and potassium permanganate) may be responsible for the formation of the dense core in granular vesicles (Coupland and Hopwood, 1968; Hökefelt and Jonsson 1968, and Solcia, Sampietro and Capella, 1969).

(k) Granular vesicles shown to reveal primary catecholamines, AD and 5-HT can be selectively demonstrated in glutaraldehyde fixed tissue by the methods described by Wood (1965, 1966) and Cannata, Chioechio and Tramezzani (1968).

There is some evidence that monoamines are stored in lysosomes or lysosome-like particles. Potter and Axelrod (1962) showed an association of H^3 noradrenaline in microsomes of the rat heart. Since then, autofluorescence has been reported to occur in brain lysosomes, and the suggestion has been made that these subcellular structures are the storage sites of monoamines (Koenig 1964). Recently van Orden, Vugman, Bensch and Giarman (1967) demonstrated by microspectrofluorometry the occurrence of 5-HT in organelles which have morphological characteristics of lysosomes.

2. MOLLUSCS

A strictly electron microscopical study of Gerschenfeld (1963) classified the synapses in the nervous systems of the slug Vagunula solea and the snail Cryptomphallus aspersa into different types. He suggested that granular vesicles, 80-110nm in diameter, contained either 5-HT or catecholamines, similar to the subcellular localisation of monoamines in the vertebrates. Accumulation of other results indicates that Gerschenfeld is likely to be correct.

Areas shown to contain monoamines by fluorescence microscopy have been studied by electron microscopy (Cottrell 1967b; Zs-Nagy 1967a). In combination with depletion experiments, Zs-Nagy (1967a) concluded that DA is located in small dense core vesicles in the bivalve Anodonta cygnea. Cottrell (1967b), moreover, applied the method described by Wood (1965, 1966) for the electron microscopic demonstration of amines in the ganglia of Spisula solida. He found granulated vesicles 30-100nm in diameter almost exclusively in the neuropile regions and suggested that these sites represent the localisation of DA and also probably of 5-HT and NA. Applying the same

technique to the ganglia of Helix aspersa, Neuman, Kerkut and Walker (1968) showed monoamines to be present in small vesicles containing a dense core.

The subcellular localisation of 5-HT in lamellibranch tissues, known to contain high amounts of the amine, was studied by density gradient (Welsh 1958; Cottrell 1965; Cottrell and Maser, 1967) and differential centrifugation (Zs-Nagy, Rózsa, Sálanki, Foldes, Forenyi and Demeter 1965). In Anodonta (Zs-Nagy et al. 1965) 5-HT was found apparently related to membranes of the endoplasmic reticulum, whereas in Mercenaria no evident association of 5-HT either with dense core vesicles described in molluscs (Gerschenfeld 1963), or with endoplasmic reticulum was found. These results are at variance with those obtained from subcellular analysis of vertebrate brain, where 5-HT was principally located in isolated nerve endings (Michaelson and Whittaker 1962 and Zieher and De Robertis 1963). Since some of the best evidence for considering 5-HT to act as a neurotransmitter comes from studies performed on molluscan neurons, it is important to make clear the precise localisation of the amine. One of

the objectives of this thesis was to discover a giant neuron (in the gastropod central nervous system) that contains only 5-HT, so that the amines true subcellular localisation could be studied, thereby resolving this question.

METABOLISM OF MONOAMINES

1 SYNTHESIS (In the vertebrates).

The main pathways for the biosynthesis of primary catecholamines are illustrated in fig 1. Goodall and Kirschner (1958) were the first to show that homogenates of sympathetic nerves or ganglia were able to convert labelled tyrosine into dopamine and noradrenaline. The first step in the hydroxylation of tyrosine is 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. This enzyme has been demonstrated in a wide variety of mammalian tissue and the properties of the enzyme have been reviewed (Undenfriend 1966). It has been suggested that the limiting step in the synthesis of primary catecholamines might be the formation of DOPA from tyrosine (Levitt, Spector, Sjoerdsma and Undenfriend 1965). The synthesis of DOPA by the enzyme DOPA decarboxylase has been reviewed by

Sourkes (1966). The reaction seems to be very efficient and proceeds at a considerable rate. DOPA decarboxylase is widely distributed in peripheral and brain tissues, known not only to contain high amounts of catecholamines but also 5-HT (Holtz and Westermann 1956). Evidence has been presented which indicates that the same enzyme is involved in the decarboxylation of DOPA and 5-HTP (5-hydroxytryptophan) to their respective amines (Rosengren 1960a and Cardot 1964a). The next step in the formation of NA is the hydroxylation of DA by the enzyme dopamine-β-hydroxylase. The localisation of this enzyme has been studied by Undenfriend and Creveling (1959) and has been reviewed by Kaufman and Friedman (1965).

The biosynthesis of 5-HT from tryptophan proceeds through two consecutive reactions; hydroxylation of tryptophan to 5-HTP, which is subsequently decarboxylated to 5-HT (see fig 2). Freedland, Wadzinski and Waisman (1961) were the first to demonstrate the enzymic hydroxylation of tryptophan with a soluble supernatant fraction obtained from rat liver. The enzyme, tryptophan-5-hydroxylase, has since been

detected in a variety of animals and the activity of the enzyme has been localised in a particulate fraction, presumably mitochondrial, of fractionated brain tissue (e.g. Gal, Armstrong and Ginsburg 1966). The properties of the enzyme have since been studied (Ichiyama, Nakamura, Nishizuka and Hayaishi 1968). Decarboxylation of 5-HTP to 5-HT by the enzyme 5-HTP decarboxylase is well established and has been reviewed by Hagen and Cohen (1966). The enzyme is distributed throughout the soluble, microsomal and crude mitochondrial fractions (De Robertis 1964).

2. STORAGE AND UPTAKE

The literature on the storage of monoamines has shown that the monoamines are stored in granules, and that the bulk of the amines in various tissues can be recovered in a particulate form following homogenisation in isotonic media. The mechanisms by which monoamines are taken up, bound and released by granules in tissues remain obscure (Iversen 1967). The observation (Hillarp 1958) that catecholamines and adenine nucleotides are found in granules in molar ratios, such that the total cationic charges of the catecholamines are balanced by anionic charges of adenine nucleotides, has led to the hypothesis that

these compounds react with an intragranular protein to form a non-diffusible complex, which last acts as a storage mechanism for the catecholamines. Remarkably high concentrations of ATP in platelets (Schmitz, Schleipen and Gross 1962) and in enterochromaffin granules (Prusoff 1961) have suggested that this nucleotide is also involved in the binding of 5-HT within the granules.

The uptake of catecholamines in the adrenal medulla (Carlsson, Hillarp and Waldeck 1963) and splenic nerve granules (Stjärne 1964) is temperature-sensitive, stimulated by Mg^{++} plus ATP, and inhibited by ethylene diaminetetraacetate (EDTA), by reserpine and other drugs, and by a number of reagents which react with sulphydryl groups. In contrast, the uptake of AD or NA in catecholamine containing particulate functions of cat heart (Potter and Axelrod 1963) and cat brain (Mirkin and Gillis 1963) is only moderately temperature-sensitive and is not stimulated by Mg^{++} plus ATP or inhibited by low concentrations of reserpine. The uptake of 5-HT by platelets depends on temperature and can be facilitated by the addition of potassium and

phosphate (or citrate) to the medium. Platelets appear to derive most of their energy from anaerobic glycolysis, but whether exogenous glucose, ATP, or other sources of energy facilitate 5-HT uptake is controversial, especially since the uptake mechanism can be inhibited by metabolic poisons such as fluoride and iodoacetate (see Maynert and Isaac 1968).

3) TURNOVER

Available evidence indicates that brain DA is turned over at a high rate. Holzer and Hornykiewicz (1959) reported that after injection of harmine, a quick acting inhibitor of MAO, the DA content of rat brain rose to a maximum level in 10-20 minutes. From this finding it seemed that the turnover of brain DA, while comparable to brain 5-HT (Undenfriend and Weissbach 1958), is distinctly higher than that of brain NA. This view is substantiated by the work of Glowinski, Iversen and Axelrod (quoted by Hornykiewicz 1966). They measured the relative rates of disappearance of H^3 dopamine and H^3 noradrenaline from the striatal tissue after the amine had been taken up from the lateral ventricle in vivo and found that DA disappeared at a faster rate than noradrenaline.

On the basis of experiments in which 5-HT content of rat brain was found to rise sharply after administration of amine oxidase inhibitors, Undenfriend and Weissbach (1958) suggested that the half-life of the amine is no longer than 30 minutes. The rapid turnover of 5-HT in brain tissue had been confirmed (Neef and Tozer 1968), suggesting that the amine serves an important role in maintaining normal brain function. A similar rate of turnover of 5-HT also occurs in the optic ganglia of Eledone moschata, where the half-life is no longer than 10 minutes (Bertaccini 1961).

4. INACTIVATION

In the vertebrates there are different ways of inactivating the 'released' monoamines:

(a) Local enzyme destruction - The enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) are important in the catabolism of monoamines in the central nervous system. This can be seen, inter alia, from the observation that the 5-HT metabolite, 5-hydroxyindoleacetic acid, and DA metabolites 3,4-hydroxyphenylacetic acid, homovanillic acid, methoxytyramine and NA metabolites normetadrenaline, 3,4-dihydroxymandelic acid and vanillinmandelic acid exist at the same locations

as the corresponding amines (e.g. Hoose 1962; Anden, Hoose and Werdinius 1963; Haggendal 1963 and Carlsson and Waldeck 1964). Cell fractionation methods have shown MAO to be located in mitochondria of nerve endings and there now seems to be general agreement that the enzyme exerts its function mainly intraneurally. COMT is also located in nerve endings, but its high degree of solubility and its lack of structural binding have made it impossible to localise. It is now thought that the enzyme is important for inactivation of extracellular monoamines (Anden, Carlsson and Haggendal 1969), although it may have a synaptic action as postulated by Axelrod (1964).

In the invertebrates the distribution of MAO activity has been studied by Blaschko and Hope (1957). Activity was found in the viscera of Patella (mollusc) and in the nervous tissue of Asterias rubens (echinoderm). No enzyme activity was detected in the hepatopancreas and gills of Cancer pagurus (arthropod) and whole animal homogenates of either Lumbricus terrestris (annelid) or Hirudo medicinalis (annelid). MAO has been found by Cardot (1964b) in the nervous ganglia of Helix pomatia, but further research by this author

(Cardot 1965) has demonstrated that this enzyme is not active on a 5-HT substrate.

(b) Membrane Pump - Recapture of the released monoamine by a 'membrane pump' appears to be an important mechanism under the normal conditions (Lundberg and Stitzel 1968). There are, however, differences between the neuronal membrane pump since they are blocked by different drugs. The membrane pump of NA neurons is inhibited by compounds of the imipramine group, whereas the membrane pump of DA neurons are unaffected by all the members of the imipramine group (Carlsson, Fuxe and Ungerstedt 1968).

(c) Diffusion away from the synaptic area - The released transmitter substance may diffuse from the synaptic gap into the blood stream. Under certain physiological conditions such as hard muscle exercise, rather high NA levels are found in the blood (Carlsson, Grimby and Häggendal 1967), and it seems likely that in these cases the 'overflow' is the main way of inactivating excess monoamines. Circulating catecholamines are metabolised mainly by liver COMT.

DRUGS THAT INTERFERE WITH MONOAMINE METABOLISM

There are a number of drugs which reduce monoamine stores e.g. reserpine, tetrabenzamine, p-chlorophenylalanine, DOPA decarboxylase inhibitors and DOPA analogues (e.g. m-tyrosine and α-methyl-m-tyrosine), while other drugs increase these stores (e.g. MAO inhibitors, COMT inhibitors and monoamine precursors). The effects of these drugs on monoamine stores vary in different animals, and it has been found necessary to study the drugs not only with respect to their dose-response and time-curves, but also with respect to their time-temperature curves. I shall consider the selective use of some drugs which were used to study the localisation of individual monoamine stores in molluscan tissues.

(1) Reserpine

Reserpine was first shown by Pletscher, Shore and Brodie (1955) to reduce levels of endogenous 5-HT. It was subsequently found that the drug was also capable of releasing NA and other amines from their stores. Numerous investigators have since shown that a single dose of reserpine can cause depletion

of catecholamines and 5-HT from brain and from peripheral sites in animals, the depletion lasting days or weeks. It is now thought that the drug depletes monoamine stores by acting on the monoamine storage granules so causing a longlasting block of their ATP-Mg^{++} dependent uptake-storage mechanism (see Carlsson 1965).

In molluscs the effect of reserpine has been investigated by Bertaccini (1961), using optic ganglia of Eledone moschata. He reported that after reserpinization the catecholamine content dropped from 1.12 to 0.12 µg/g. This result was substantiated by Dahl, Falck, Mecklenburg, Myhrberg and Rosengren (1966), who showed that large doses of the drug (10mg/Kg daily for two days or one injection of 30-50 mg/Kg) are necessary to cause the complete disappearance of monoamine fluorescence in Anodonta piscinalis and Helix pomatia. Mirolli and Welsh (1964) showed that the time course for the lowering of 5-HT levels in molluscan ganglia, by reserpinization, is dependent on temperature.

2. α-Methyl-m-tyrosine.

The drug α-methyl-m-tyrosine depletes catecholamine stores in inhibiting the enzyme tyrosine hydroxylase (Spector, Sjoerdsma and Undenfriend 1965),

so preventing the metabolism of primary catecholamines. The time course of the action of α-methyl-m-tyrosine, in guinea pig brain tissue, is such that at 4 hours depletion of both NA and DA is maximal, while at 17 hours NA is still lowered and recovery of DA is almost complete (Moore 1966). The drug has no effect upon the 5-HT concentration.

(3) p-Chlorophenylalanine

p-Chlorophenylalanine has been shown to be a potent and selective depleter of 5-HT in vertebrate tissues, including the brain. The drug acts by inhibiting the hydroxylation of tryptophan, the first rate-limiting reaction in the synthesis of 5-HT (Koe and Weissman 1966). Bloom and Giarman (1967) have observed that p-chlorophenylalanine (316 mg/Kg) decreases the 5-HT content of the pineal gland in rats by more than 90% within a day without affecting NA content. 5-HT in the pineal gland returns to normal levels by 5 days after treatment.

(4) DOPA decarboxylase inhibitors

Reduction of catecholamine stores can be brought about by drugs which inhibit the enzyme which decarboxylates DOPA to DA. Since experimental evidence

shows DOPA decarboxylase and 5-HTP decarboxylase to be the same enzyme (see Rosengren 1960a and Cardot 1964a), an administered decarboxylase inhibitor should reduce both catecholamine and 5-HT contents. A number of decarboxylase inhibitors have been used, but results have been confusing. According to the present data, the decarboxylase inhibitor, NSD 1024 (m-hydroxybenzyloxyamine) reduces rat brain NA content but has little effect upon the 5-HT concentration. It has been suggested that the decarboxylating enzyme is present in greater quantity than is necessary for its function, which may explain why decarboxylating inhibitors often have little effect upon normal monoamine synthesis (Carlsson 1964). The brain of snails, pretreated with NSD 1024, showed a reduction in catecholamine content and no effect on 5-HT concentration (Kerkut, Sedden and Walker 1967).

(5) Monoamine Precursors

In contrast with monoamines themselves, their precursors, once injected into the animal, are able to penetrate into the brain where they undergo decarboxylation to their respective amines, i.e. DA (from DOPA),

5-HT (from 5-HTP) and DA (from 3,4-dihydroxyphenylserine and DOPA). Certain objections have been raised against the use of precursors, because experiments in vitro indicate that some neurons take up both catecholamine and 5-HT precursors. (Costa, Cessa, Kuntzman and Brodie 1962). Since 5-HTP decarboxylase and DOPA decarboxylase is now thought to be the same enzyme, it would mean, for example, that after injection of 5-HTP, 5-HT would accumulate not only in seretonergic synapses but also at noradrenergic, dopaminergic and perhaps adrenergic synapses. However, in mammals, the syndromes produced by 5-HTP and DOPA are markedly different, indicating that different receptors are activated by their respective decarboxylation products. In practice it has been found that catecholamine and 5-HT neurons have a fairly high specificity to take up (and/or decarboxylate) DOPA and 5-HTP respectively. (Fuxe, Dahlström and Hillarp 1965).

In the molluscs, Bertaccini (1961) found that administration of DOPA increased the DA content of the optic ganglia of Eledone moschata from 1.2 to 3 µg/g within an hour. Similarly, Cardot (1963) showed that injection of DOPA into gastropod molluscs led to a rise in brain DA levels, and Welsh and Moorhead (1959)

observed that administration of 5-HTP led to an increase of 5-HT content in gastropod brain. Using fluorescence microscopy for locating amines, Kerkut, Sedden and Walker (1967) demonstrated that administration of DOPA to snails results in a marked increase in amine levels of catecholamine cell bodies, but not in 5-HT perikarya. Similarly, injection of 5-HTP caused an increase of amine in 5-HT cells while the catecholamine neurons were not affected. Experiments in vitro have shown that the enzyme responsible for the decarboxylation of 5-HTP is present in neurons and not in the connective sheath of gastropod ganglia (Mirolli 1968).

(f) Inhibitors of MAO and COMT.

Since MAO is involved in the catabolism of monoamines (see figs 1 and 2), inhibition of the enzyme leads to an increase in all catecholamine and 5-HT levels. Iproniazid inhibits MAO irreversibly and non-competitively, and is very effective in the brain but not effective elsewhere (Undenfriend, Weissbach and Bogdanski 1957). In contrast nialamide inhibits MAO in the central and peripheral nervous systems. Injection of nialamide into mice causes a greater increase of NA and/or DA than 5-HT (Carlsson 1964).

MAO inhibitors are effective in the molluscs. Bertaccini (1961) has recorded a rise in catecholamine level in the optic ganglia of Eledone moschata after treatment with iproniazid and harmoline. Also Zs-Nagy (1967b) found that iproniazid increases the intensity of green monoamine fluorescence in Anodonta cygnea, Dahl, Falck, Mecklenburg, Myhrberg and Rosengren (1966) showed that injection of nialamide into Anodonta piscinalis produces an increase of monoamine content in nerve fibres but not in their cell bodies.

The enzyme COMT seems to be responsible for the breakdown of monoamines (see Figs 1 and 2), although its role is not certain, probably due to a lack of selective inhibitors of the enzyme. Of all inhibitors available pyrogallol has been used the most extensively. A striking rise in the concentration of catecholamine has been recorded in the brain of rabbit after administration of the inhibitor (Weil-Malherbe, Posner and Bowles 1961). Unfortunately pyrogallol is toxic and produces many reactions unrelated to COMT inhibition, so its use is limited. Administration of quercetin, another COMT inhibitor, prevents the disappearance of ^{3}H-adrenaline from mice, so that the amount of labelled material in the animal is eventually doubled. (Axelrod and Tomchick 1959). Derivatives of 3,4-dihydroxy-

phenylacetamide are known to cause noticeable inhibition of COMT *in vivo*, but their side effects are still being investigated (Carlsson 1964).

MONOAMINES AS NEUROTRANSMITTERS IN MOLLUSCA

1) EVIDENCE FOR 5-HT AS A CARDIO-EXCITATORY TRANSMITTER.

As early as 1953 Welsh and Moorhead showed that 5-HT has a pronounced excitatory effect on *Mercenaria mercenaria* heart and suggested that the agent might be the excitatory transmitter substance normally released from cardiac nerves. A lot more data in agreement with this view have been obtained since then:

(a) 5-HT and its immediate precursor 5-HTP are present in molluscan nervous systems; the enzyme for conversion of 5-HTP to 5-HT and for the subsequent inactivation of 5-HT have been detected in molluscan tissue. (see review of Literature).

(b) Kerkut and Cottrell (1963) showed chromatographically that the snail heart (*Helix aspersa*) contains an appreciable amount of 5-HT (3 μg/g).

(c) Stimulation of the cardio-excitatory nerve results in either an inhibitory or excitatory effect on the heart, depending on the amplitude and frequency of stimulation.

In lamellibranchs, Welsh (1957) and Loveland (1963) established a comparison between the effect of 5-HT and the excitatory effects on the heart from stimulation of the cardio-excitatory nerve. Koshtoyants (1957) arrived at similar conclusions for gastropods.

(d) Hearts of animals previously treated with reserpine, showed decreased excitatory effects on stimulation of the cardio-excitatory nerve. This was ascribed to the reduced amounts of 5-HT available in the nerve endings, since the sensitivity of the hearts to applied 5-HT was unaffected (Loveland 1963).

(e) Lysergic acid derivatives BOL and UML (Loveland 1963) and chlorpromazine (Rózsa and Graul 1964) block the response of the heart to stimulation of the cardio-excitatory nerve and to applied 5-HT. Since BOL affects nerve conduction (Gerschenfeld, Ascher and Tauc 1966) in Aplysia, the value of this drug in testing serotonergic transmission in molluscs needs to be re-examined.

(f) Loveland (1963) showed that previous injection of MAO inhibitor iproniazid into Mercenaria resulted in an enhancement of excitation of the heart on stimulation of the cardio-excitatory nerve.

(g) Mercenaria heart made tachyphylactic to 5-HT failed to respond to stimulation of the cardio-excitatory nerve.

Subsequent washing for a suitable period of time however, restored the sensitivity of the heart to both stimulation of the nerve and to applied 5-HT (Loveland 1963).

(h) By arrangement of donor and recipient hearts, similar to that devised by Loewi (1921), Rózsa and Graul (1964) showed that heart excitation resulted from the release of a chemical from the cardio-excitatory nerves on stimulation.

(j) 5-HT has been detected chromatographically and spectrophotofluorometrically in heart perfusates of Helix pomatia, following stimulation of the cardio-excitatory nerve, but not in perfusates from unstimulated hearts (Rózsa and Perényi 1966).

(2) EVIDENCE FOR 5-HT AS AN EXCITATORY TRANSMITTER IN THE CENTRAL NERVOUS SYSTEM.

CILDA cells are neurons in the brain of gastropods which, when stimulated presynaptically, exhibit an inhibition of long duration (Gerschenfeld and Tauc 1964). A lot of evidence has been presented by Gerschenfeld and Stefani (1966, 1968) and Stefani and Gerschenfeld (1969), supporting the idea that 5-HT could be the chemical transmitter responsible for the excitation of CILDA neurons:

(a) As would be expected, if 5-HT acts as a transmitter on the CILDA cell, the amine increased membrane conductance by about 40%.

(b) Very low concentrations of 5-HT always depolarised and excited CILDA cells. It was calculated that only 8.2×10^{-9} moles of 5-HT (an order expected from a natural transmitter substance) resulted in a depolarisation of 4 mv.

(c) Repeated application of 5-HT produced intense desensitisation similar to that seen from ACh receptors on cells known to receive a cholinergic input: e.g. as in the vertebrate muscle end-plate, and in the H- and D-cells in the abdominal ganglion of Aplysia dipilans (Gerschenfeld and Tauc 1961).

(d) 5-HT has been biochemically detected in gastropod central ganglia and located by histochemical methods in neuronal somata and in both axons and nerve endings at the neuropile (page 18).

(e) The presence of enzymes capable of decarboxylating 5-HTP has been demonstrated in snail ganglia (Kerkut and Cottrell 1963; Cardot 1963; and Mirolli 1968).

(f) Concentration of MAO inhibitors (iproniazid and transylcypromine) greater than 5×10^{-3}M, blocked

response of CILDA cells to 5-HT, but in weaker concentrations, transylcypromine enhanced the 5-HT response. Gerschenfeld and Stefani suggested that the potentiation of 5-HT might have resulted from inhibition of MAO in the mitochondria, or from transylcypromine acting upon 5-HT receptors.

(g) 5-HT can be specifically blocked by LSD (10^{-4} g/ml) tryptamine (10^{-5} g/ml) and 5-HT itself (10^{-5} g/ml). These drug concentrations did not affect ACh receptors present on the membrane of the same cells. Hexamethonium bromide (10^{-5} g/ml) which blocked the ACh receptors, did not affect the 5-HT receptors.

(h) Gerschenfeld and Stefani have concluded from their studies that 5-HT is the chemical transmitter responsible for the slow excitatory post synaptic potentials of CILDA neurons.

3) EVIDENCE FOR DA AND NA AS TRANSMITTER SUBSTANCES IN THE CENTRAL NERVOUS SYSTEM.

Pharmacological studies on individual central neurons of gastropods suggest that DA and possibly NA act as transmitter substances in this localization. The evidence for this is as follows:

(a) Certain gastropod neurons are pharmacologically very sensitive to DA (Kerkut and Walker 1962; Gerschenfeld 1964; Ascher, Kehoe and Tauc 1967; Ascher 1968 and Walker, Woodruff, Glaizner, Sedden and Kerkut 1968) and NA (Glaizner 1967, 1968).

(b) The effect of DA on different cells varies when applied electrophoretically. Some cells are depolarised, others are hyperpolarised (Walker, Woodruff, Glaizner, Sedden and Kerkut 1968) whereas a few show biphasic effects (Ascher 1968).

(c) Of all the naturally occurring substances tested on D-inhib cells (so designated by Gerschenfeld (1964) because application of ACh results in depolarization or excitation, and furthermore because the cells receive a non-cholinergic inhibitory input), DA was the most active inhibitor. The α-adrenergic blocking agents dibenamine and dihydroergotamine did not affect the inhibitory input.

(d) NA applied to cells produces only inhibitory effects (Glaizner 1967, 1968).

(e) Experiments with blocking agents (Walker, Woodruff, Glaizner, Sedden and Kerkut 1968, Glaizner 1967,1968)

confirmed that the membrane of some cells have DA and/or NA receptors, Only α-adrenergic blocking substances antagonised the response to DA while the β-blocker propranolol was ineffective. On the other hand propranolol completely blocked the response to applied NA.

(f) Woodruff and Walker (1969) worked out the structural requirements of DA receptors in snail neurons, which consist of hydroxyl groups on the 3 and 4 positions of the benzene ring and the presence of a terminal nitrogen either unsubstituted or with one methyl group.

(g) DA has been shown by biochemistry to occur in snail central ganglia, and primary catecholamines have been located by histochemical methods in perikarya and in both axons and nerve endings in the neuropile (see page 20).

(h) The presence of enzymes capable of decarboxylating DOPA has been demonstrated in the central nervous system of gastropods (Cardot 1963; 1964; Kerkut, Sedden and Walker 1966, and Sweeney 1969).

PLAN OF INVESTIGATION

As I have already mentioned, (see page 13) large quantities of DA and 5-HT are to be found in the nervous systems of molluscs. It was decided to investigate the occurrence of these substances in the gastropods, bearing in mind the role suggested for them as transmitter agents.

The initial step was to examine the presence of monoamine catabolites in extracts of heart and brain tissue of the snail with chromatographic and spectrophotometric methods. Having detected MA catabolites, the precise monoamines present in the two tissues were analysed by applying chromatographic, spectrophotometric and biological assay techniques.

In order to localise the monoamines in heart tissue, conventional histology, viz. methylene blue and silver staining, was used to study the intrinsic innervation. In conjunction with some electron-microscopic studies, a neurosecretory system was discovered to terminate in the heart of Helix pomatia.

The next step was concerned with the cellular localisation of monoamines in different

tissues of the slug Limax maximus, and the heart of the snail Helix pomatia by the fluorescence microscopy technique. Having established the presence of monoaminergic nerves in the heart, I then investigated the effect of some drugs on the monoamine fluorescence.

On the basis of earlier results, I discovered an identifiable giant neuron containing 5-HT which could be repeatedly located in the cerebral ganglion of the slug. The amine content was investigated by using a biological assay method, and its subcellular localization by employing the technique of electron microscopy cytochemistry.

The last section of the experimental work deals with an investigation into the accumulation of monoamines in the ligatured visceral nerve of the snail Helix pomatia.

EXPERIMENTAL INVESTIGATIONS

SPECIES OF GASTROPODS AND TYPE OF SALINE USED

The garden snail Helix aspersa and the slug Limax maximus were collected locally or obtained from L. Haig and Co. Ltd., Newdigate, Surrey. Specimens of the edible snail Helix pomatia were also supplied from the dealers. On arrival at the laboratory, animals were either used immediately or kept at 9°C in a moist mixture of grass and sand containing food in the form of slices of carrots and potatoes.

The choice of saline is of consequence, especially when maintaining an isolated snail heart for long periods. For this purpose Meng (1960) saline was chosen. It contained:

NaCl	3.45 g/litre
KCl	0.43 g/litre
$CaCl_2$	1.17 g/litre
$NaHCo_3$	1.00 g/litre
$MgCl_2$	1.55 g/litre

DEMONSTRATION OF MONOAMINE METABOLITES IN BRAIN AND HEART OF HELIX ASPERSA

INTRODUCTION

The occurrence of monoamine metabolites in the vertebrates has been reviewed by Eichler and Farah (1965), Glowinski and Baldessarini (1966) and Hornykiewicz (1966). Catecholamines are inactivated by two main enzymes: (see Figs 1 and 2) Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) (see fig 1 and 2). The action of either or both of these, on DA results in the formation of 3,4-dihydroxyphenylacetic acid, methoxytyramine and homovanillic acid, respectively. Similarly, in the case of NA the corresponding products are formed : 3,4-dihydroxymandelic acid, normetadrenaline and vanillinmandelic acid. Monoamine oxidase also breaks down 5-HT to form 5-hydroxyindoleacetic acid. Other metabolites of the indolealkylamine include 5-hydroxytryptophol and melatonin.

Very little information is available on the transformation of monoamines in invertebrates, with the exception of the somewhat special case of DA, in the insect cuticle. In Calliphora Sekeris and Karlson (1966)

have reported that most of the DA present in the cuticle is acetylated to N-acetyl dopamine and used in the process of sclerotization after being oxidised to the quinone. N-acetyldopamine is also stored as the N-acetyldopamine-4-O-glycoside. A minor route of DA metabolism is oxidative deamination leading to the formation of 3,4-dihydroxyphenylacetic acid. In the protozoan Crithidia fasciculata, Janakidevi, Dewey and Kidder (1966) have observed the NA metabolites normetadrenaline and vanillinmandelic acid. Recently Adiyedi (1969) described the occurrence of another NA product, 3,4-dihydroxy-mandelic acid, in the mushroom gland of the cock-roach Periplaneta americana.

Studies were designed to detect possible phenolic acid metabolites of monoamines in the brain and heart of the snail Helix, to provide information concerning the metabolism of amines in the gastropods. Since Eccleston, Moir, Reading and Ritchie (1966) showed that both human and rat brain homogenates convert most of their 5-HT into 5-hydroxytryptophol, it was decided to investigate the occurrence of the alcohol metabolite.

METHODS

1. Extraction of phenolic acids and 5-hydroxytryptophol

Double distilled deionised water was used thoughout these experiments and the reagents were of Analar grade.

The brains (the ganglia and connectives comprising the circumoesophageal nerve rings) and hearts of 100 to 200 animals were all dissected in 8 to 12 ml of ice cold Meng's snail saline, and homogenised. The homogenates were deproteinised with 1 to 1.5 ml of 10% $ZnSO_4$ and 1 to 1.5 ml of 10% NaOH. After 3 to 5 minutes centrifugation at 3000g, the supernatants were transferred to glass-stoppered tubes. Each sample was acidified to pH2 with concentrated HCl, and then saturated with NaCl. The mixtures were extracted twice, shaken each time for 5 min., with double their volumes of ethyl acetate and centrifuged at 3000g for 10 min. The ethyl acetate extracts were shaken with 6.5 ml of 0.1M Tris buffer (trishydroxymethylaminomethane) pH 8.6 for 10 mins. After centrifugation at 3000g for 10 mins, the aqueous phase was seperated and used for the fluorometric and chromatographic detection of phenolic acid. The ethyl acetate phase was used for the chromatographic

localisation of 5-hydroxytryptophol (Ashcroft, Crawford, Dow and Guldberg 1968).

2) Spectrophotofluorometric detection of phenolic acids.

0.5 ml aliquots of each of the aqueous phases were used for analysis in an Aminco-Bowman Spectrophotofluorometer. Blanks were prepared by substituting water for the extracts in the different processes. Standards were processed concurrently.

(a) Homovanillic acid was estimated by the method of Anden, Roos and Werdinius (1963) : 0.5 ml of 5N ammonia solution and 0.1 ml of 0.01% ferricyanide were added to samples of extract. After 4 min, 0.1 ml of 0.1% cysteine was added. The samples were then read within 10 minutes at an activation wavelength of 325 mμ and an emission wavelength of 430-435mμ.

(b) 3,4-Dihydroxyphenylacetic acid was estimated by the method of Rosengren (1960) : 0.3 ml of freshly prepared mixture containing 1 part redistilled ethylene diamine and 3 parts of 4M ammonium chloride was added to each sample of extract, and these were then incubated for 20 minutes at 50°C and shaken in a stoppered tube covered with aluminium foil to exclude light. After cooling, fluorescence was measured at 530mμ with activation light at 425mμ wavelength.

(c) 5-Hydroxyindoleacetic acid was estimated by the method of Ashcroft and Sharman (1962) : 0.3 ml concentrated HCl containing ascorbic acid (0.5 mg/ml) was added to samples of extracts. Fluorescence was measured at 550 mμ with activation light at 310 mμ wavelength.

3) Chromatography

(a) Detection of phenolic acids

The remainder (5 to 5.5 ml) of each of the aqueous phases was used for chromatography. Each of the samples was acidified to pH2 with concentrated HCl, saturated with NaCl, and extracted twice with 10 ml of ethyl acetate. To each of the combined ethyl acetate extracts anhydrous sodium sulphate was added to remove any water. The supernatant solution was evaporated to dryness under reduced pressure at 5°C. Each residue was dissolved in 0.1 ml methanol and applied to a silica gel G (Merck) thin layer chromatography plate. The following standards were routinely added to the plates : homovanillic acid, 3,4-dihydroxyphenylacetic acid, p-hydroxyphenylacetic acid, p-hydroxymandelic acid and 5-hydroxyindoleacetic acid. All these compounds were obtained from Sigma Co. Ltd. Two solvent systems used were : (a) The

organic phase of chloroform/acetic acid/water (2:2:1 by volume) and (b) benzene/acetic acid/ water (125:72:3).

Phenolic acids were localised on the chromatograms by spraying with the following reagents (a) Diazonised p-nitroaniline (for phenolic acids), (b) potassium ferricyanide-ammonium hydroxide reagent (for detecting homo-vanillic acid under U.V. light), (c) 2,6-dichlor-benzoquinone-4-chloroimide (for detecting mandelic acids), and (d) HCl (for detecting 5-hydroxy-indoleacetic acid under U.V. light) (for details see Ashcroft, Crawford, Dow and Guldberg 1968).

(b) Detection of 5-hydroxytryptophol.

The ethyl acetate phases were evaporated to dryness under reduced pressure at 5°C. Each residue was dissolved in 0.1 ml 80% methanol and applied to a silica gel G (Merek) thin layer chromatography plate. To this was added a standard solution of 5-hydroxytryptophol obtained from Regis Chemical Co. The same two solvents used to separate phenolic acids were employed. 5-Hydroxy-tryptophol was localised by exposing chromatograms to iodine vapour.

RESULTS

(1) Phenolic acids

(a) Spectrophotofluorometry

The spectrophotofluorometric studies provided evidence for the presence of homovanillic acid and 3,4-dihydroxyphenylacetic acid, and for the absence of 5-hydroxyindoleacetic acid in both heart and brain extracts. The fluorescence spectra of purified heart and brain extracts are shown in figs 3 and 4. The concentration of each of the DA metabolites was greater in the brain extracts.

(b) Chromatography

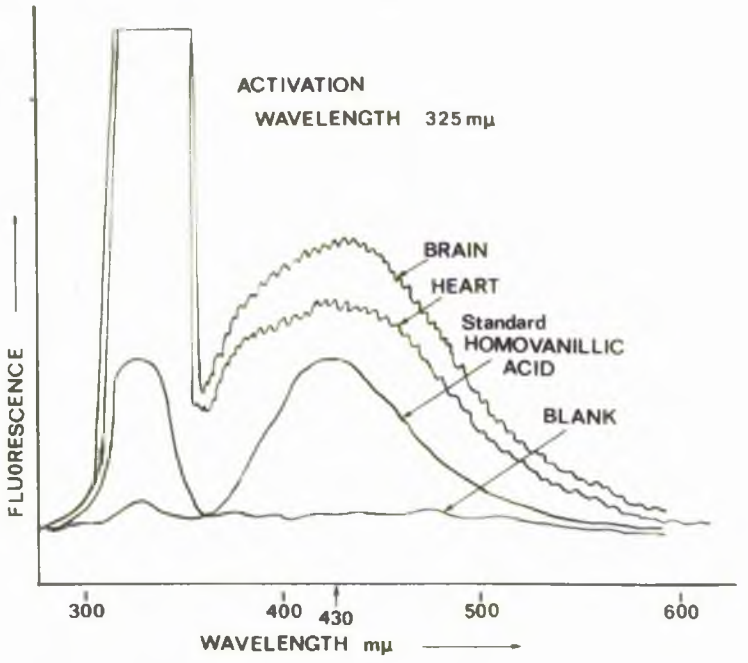
0.5 to 1 μg of each acid could be detected on the thin layer chromatograms. Both solvents gave satisfactory separation of acids. The Rf values of the phenolic acids tested, and their responses to the different reagents are summarised in Table 1.

The following observations and conclusions were made after examining chromatograms of heart and brain extracts (see fig 5). There was a substance present in both extracts which corresponds to the Rf value of homovanillic acid. The blue fluorescence observed under UV light after spraying with ferricyanide reagent was more intense in the brain than in

Fig 3. Fluorescence spectra of heart and brain extracts prepared for detecting homovanillic acid.

Fig 4. Fluorescence spectra of heart and brain extracts prepared for detecting 3,4-dihydroxyphenylacetic acid.

3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BRAIN
HEART
Standard
HOMOVANILLIC
ACID
BLANK
FLUORESCENCE
300
400
430
500
600
WAVELENGTH mμ

4

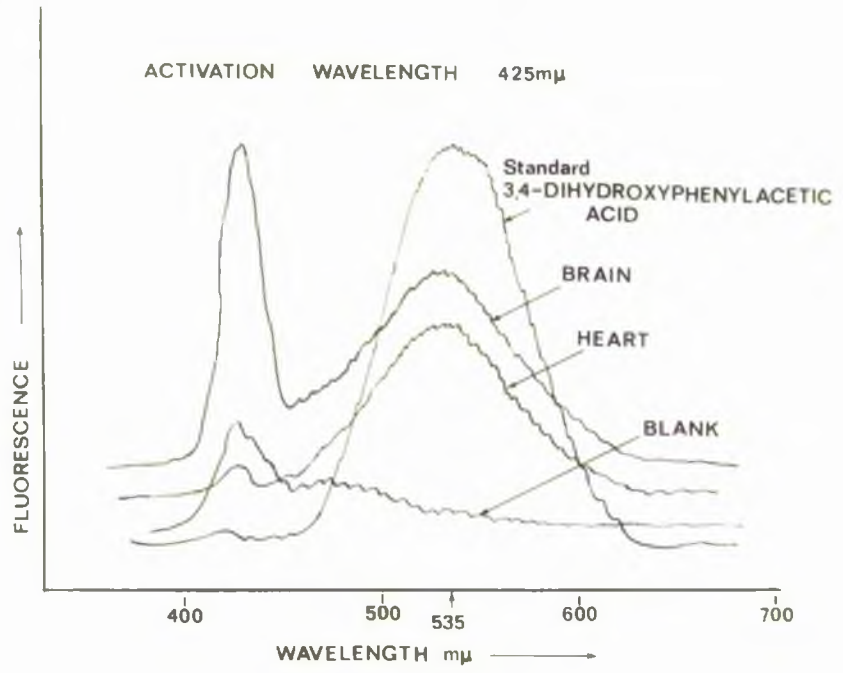

ACTIVATION WAVELENGTH 425mμ
Standard
3,4-DIHYDROXYPHENYLACETIC
ACID
BRAIN
HEART
BLANK
FLUORESCENCE
400
500
535
600
700
WAVELENGTH mμ

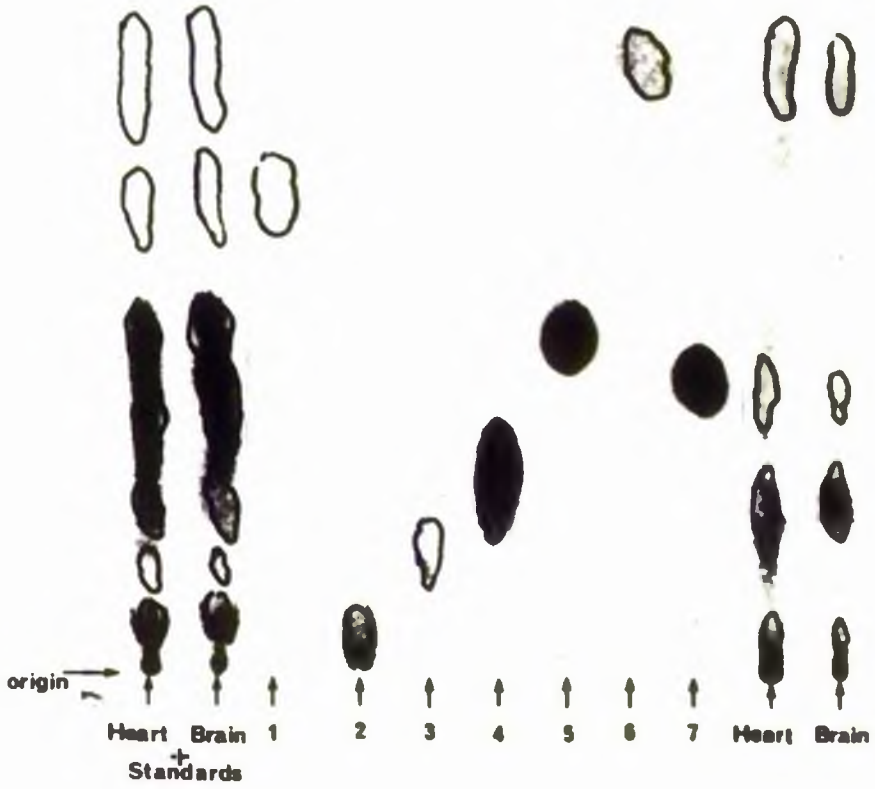
Table 1. Identification of phenolic acids on Silica gel G chromatograms as based on Rf value and coloration.

TABLE 1

COMPOUND	Rf value for chloroform/acetic acid/water solvent	After treatment with p-nitroaniline	After treatment with ferricynide reagent	After treatment with 2,6-dichlorobenzoquinone-4-chloroimide	After treatment with hydrochloric acid
Homovanillic Acid	0.75	grey/red colour	bright blue fluorescence		
3,4-Dihydroxyphenyl acetic acid	0.24	light brown	brown colour		
5-Hydroxy-3-indole acetic acid	0.27	grey/brown			pink fluorescence
p-Hydroxyphenyl acetic acid	0.56	light brown		light brown	
p-Hydroxymandelic acid	0.09	brown		purple blue	
[illegible]inmandelic acid	0.20	dark brown		dark grey/blue	
3,4-Dihydroxymandelic acid	0.03	grey		deep brown	

Fig 5. A photograph of a thin layer chromatogram of ethyl acetate extracts of heart and brain (150 snails used) after development with the chloroform/acetic acid/water solvent and spraying with p-nitroaniline. Standards used were : (1) p-hydroxyphenylacetic acid; (2) 3,4-dihydroxymandelic acid; (3) p-hydroxy-mandelic acid; (4) vanillinmandelic acid; (5) 5-hydroxyindoleacetic acid; (6) homovanillic acid; (7) 3,4-dihydroxy-phenylacetic acid.

5



the heart extracts. Thus the brain contained a greater concentration of homovanillic acid. Both heart and brain extracts also contained a substance which ran to the same position on chromatograms as 3,4-dihydroxyphenylacetic acid. Reaction with p-nitroaniline was more intense with the brain extract, which therefore had the higher concentration of this acid. Heart extract chromatograms also showed a substance with the same Rf value as vanillinmandelic acid, which, like vanillinmandelic acid, appeared light brown after spraying with p-nitroaniline, and blue after spraying with 2,6-dichlorobenzoquinone-4-chloroimide. Also present in heart extracts was a substance which corresponded to 3,4-dihydroxy-mandelic acid in Rf value and in its coloration after spraying with p-nitroaniline and also after spraying with 2,6-dichlorobenzoquinone-4-chloroimide. Fainter spots, which correspond in Rf value to the last two named phenolic acids, and which also produced characteristic colours after reaction with p-nitroaniline and 2,6-dichlorobenzoquinone-4-chloroimide, indicated that lower concentrations of

vanillinmandelic acid and 3,4-dihydroxymandelic acid were present in the brain. These data therefore suggested the presence of both acid metabolites of DA and NA in the brain and heart, and indicated that the brain contained a higher concentration of the DA products, whereas the heart had greater content of NA products. In addition, from the observation that the brain contains 3,4-dihydroxymandelic acid and vanillinmandelic acid it would seem that NA is formed in the brain.

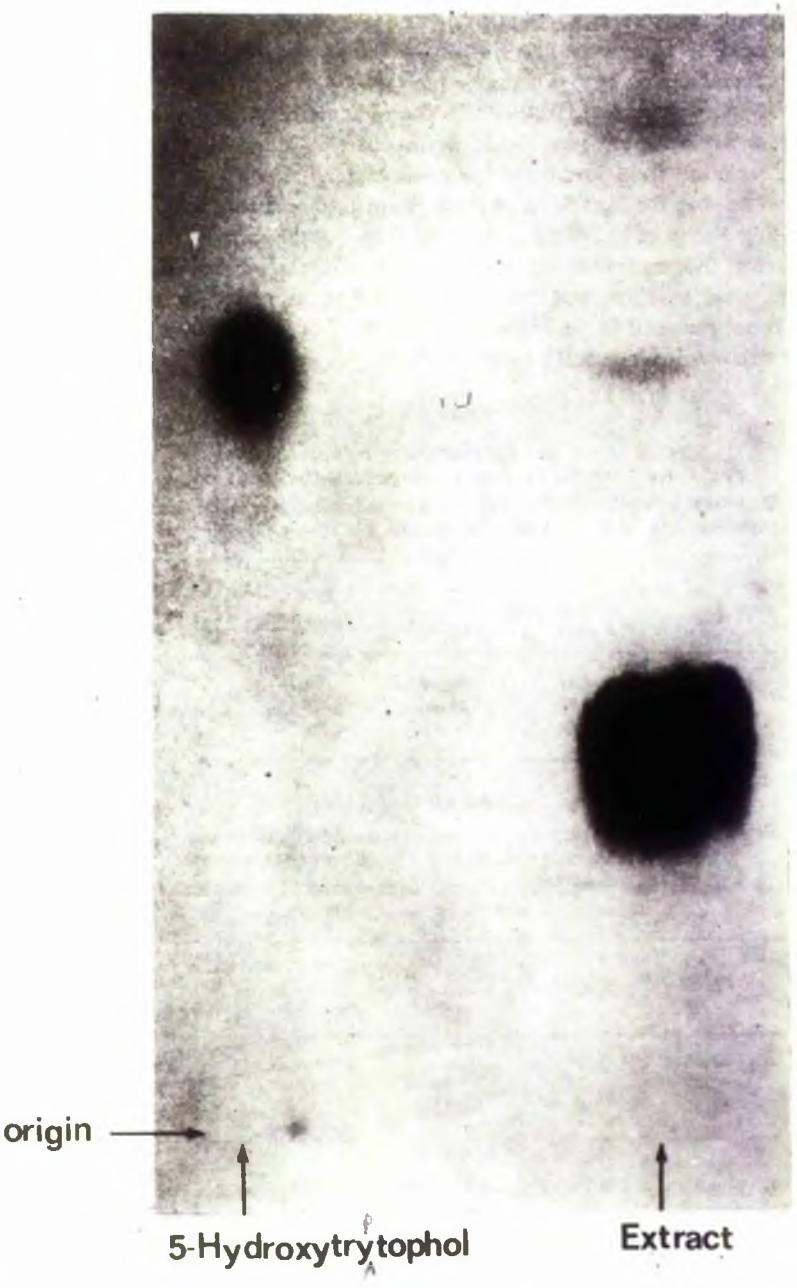
There was no evidence for the presence of 5-hydroxyindoleacetic acid, p-hydroxymandelic acid or p-hydroxyphenylacetic acid in either of the two tissue extracts.

(c) 5-Hydroxytryptophol

There was a substance in chromatograms of heart and brain extracts corresponding to the Rf value of pure 5-hydroxytryptophol. Faint light brown material, after exposure to iodine vapour, was more intense in the brain than the heart extracts (fig 6). This result suggested the occurrence of the alcoholic metabolite of 5-HT in brain and heart and a higher concentration in the brain. In both cases the amount of 5-hydroxytryptophol present was very low.

Fig 6. Photograph of a thin layer chromatogram of brain extract and standard (5-hydroxytryptophol) after development with the chloroform/acetic acid/water solvent and exposure to iodine vapour.

6



CONCLUSIONS

1. Using a combination of spectrophotofluorometry and thin layer chromatography, the phenolic acids 3,4-dihydroxyphenylacetic acid, homovanillic acid, 3,4-dihydroxymandelic acid and vanillinmandelic acid were detected in both heart and brain extracts of Helix aspersa. There was no evidence for the presence of 5-hydroxyindoleacetic acid.

2. Greater quantities of NA products were present in the heart than the brain, whereas the brain contained the higher levels of the DA metabolites.

3. Thin layer chromatography provided evidence for the occurrence of small amounts of 5-hydroxy-tryptophol in brain extracts and to a lesser extent in heart extracts.

4. p-hydroxyphenylacetic acid and p-hydroxymandelic acid, both possible metabolites of tyramine and octopamine respectively, were not detected in tissue extracts studied.

ESTIMATION AND DETECTION OF MONOAMINES IN HEART AND BRAIN TISSUES OF HELIX POMATIA

INTRODUCTION

The localization of NA metabolites in heart and brain extracts prompted me to investigate the possible occurrence of NA, and to estimate the quantity of each monoamine present in these tissues.

It was also decided to estimate separately the content of 5-HT in auricle and ventricle extracts to see whether the amine has a uniform distribution, for example, in muscle cells as suggested by Rózsa and Zs-Nagy 1967.

METHODS

(1) Chromatographic analysis of Monoamines.

Freshly dissected brains (the ganglia and connectives comprising the circumoesophageal nerve rings) and hearts from 100-150 animals were homogenised in solutions of ice cold 50% acetone in 0.01N HCl and centrifuged for 30 minutes at 50,000g. Supernatant solutions were freeze dried, resuspended in a small volume of acetone/HCl and applied to paper (Whatman 3mm) or thin layer chromatograms (Chromedia-Merck), which were subsequently developed in an ascending fashion. The solvent systems used were: Phenol/HCl/KCN

(Waalkes, Sjoerdsma, Creveling, Weissbach and Undenfriend 1958) for paper chromatograms, and n-butanol/HCl (Bertler and Rosengren 1959) for paper and thin layer chromatograms. The amines were detected with paraformaldehyde according to the method of Bell and Somerville (1966).

The monoamine content of chromatographed spots was estimated by comparison with known amounts of pure substance (ranging in amount from 0.05-1.5µg amine for paper chromatography) by direct vision under ultraviolet light (peak emission 254mµ and 366mµ).

(2) Pharmacological detection and estimation of NA

A strip of muscle of the fundus part of the rat stomach was prepared as described by Vane (1957). The muscle strip was suspended to a 10 ml bath fitted with Krebs' solution, which contained 1 to 15 µg of 5-HT per litre (Armitage and Vane 1964). The temperature of the bath was kept at 37°C. Areas of unstained paper chromatograms corresponding to the Rf value of standard NA were thoroughly dried, eluted with Krebs' solution, and tested on the isolated strip of muscle.

In other experiments, 15 to 20 µg/litre of 5-HT, DA and ACh were added to the saline solution, Crude extracts were prepared by homogenising tissue from 12 to 20 animals in Krebs' solution, and tested on the isolated muscle.

(3) Spectrophotofluorometric estimation of 5-HT in auricle and ventricle of heart.

Double distilled deionised water and Analar grade reagents were used throughout these experiments.

The auricles and ventricles of from 25 to 50 animals were each dissected into 0.3 to 0.6 ml of ice cold 0.1N HCl containing 0.5% ascorbic acid, and then homogenised. Extraction and estimation of 5-HT in extracts, standard solutions of 5-HT, and blanks, were carried out by the following method of Wilhoft and Quay (1965).

The first step in the extraction was to wash each sample with diethyl ether containing NaCl and ascorbic acid in EDTA (ethylenediaminetetra-acetic acid) and then to transfer the amine into alkaline borate buffer. Next, the 5-HT was transferred to butanol buffer phase. Finally the 5-HT was conveyed back into the aqueous phase by shaking with heptane containing 0.1N HCl ascorbic acid and removing the organic phase.

RESULTS

(1) Chromatography

About 0.01 μg of primary catecholamine and 0.02 μg of 5-HT could be detected, using thin layer chromatography. Paper chromatography, although less sensitive than thin layer chromatography had the advantage of separating components of the extracts more clearly. The Rf values for the different substances applied to chromatograms are shown in Table 2.

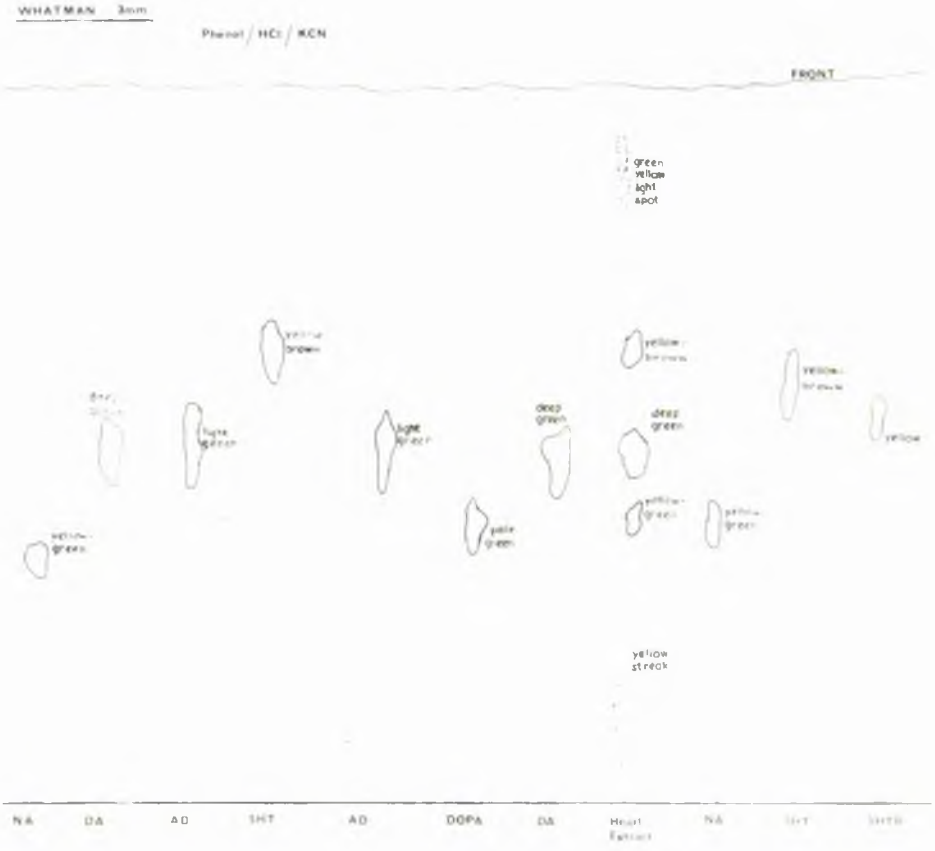
With chromatograms of brain extracts, relatively intense spots corresponding to the Rf values of DA and 5-HT were observed. A distinct spot was not observed, however, at the Rf value of NA. Any faint spot in this position would have been obscured by a long streak of unidentified fluorescent material.

Extracts of heart tissue were generally clearer than those of the brain. Chromatograms of heart extracts showed three distinct fluorescent spots which correspond in position and colour to DA, NA and 5-HT (Fig 7 and 8). The fluorescence intensities of the compounds indicate that the concentrations of DA and NA were of the same order, and that each was greater than the concentration of 5-HT.

Fig 7. Trace of a developed paper chromatogram of heart extract showing the positions and colour of fluorescent spots observed under U.V. light.

Fig 8. Photograph of a developed thin layer chromatogram of heart extract showing the fluorescent spots observed under U.V. light. Identification of the extract spots was based on Rf value and coloration of fluorescence (see Table 2).

7


WHATMAN 3mm
Phenol / HCl / KCN
FRONT
green
yellow
light
spot
yellow
brown
light green
light green
deep green
deep green
yellow
green
pale green
yellow
green
yellow
green
yellow
streak
NA
DA
AD
5HT
AD
DOPA
DA
Heart Extract
NA

8

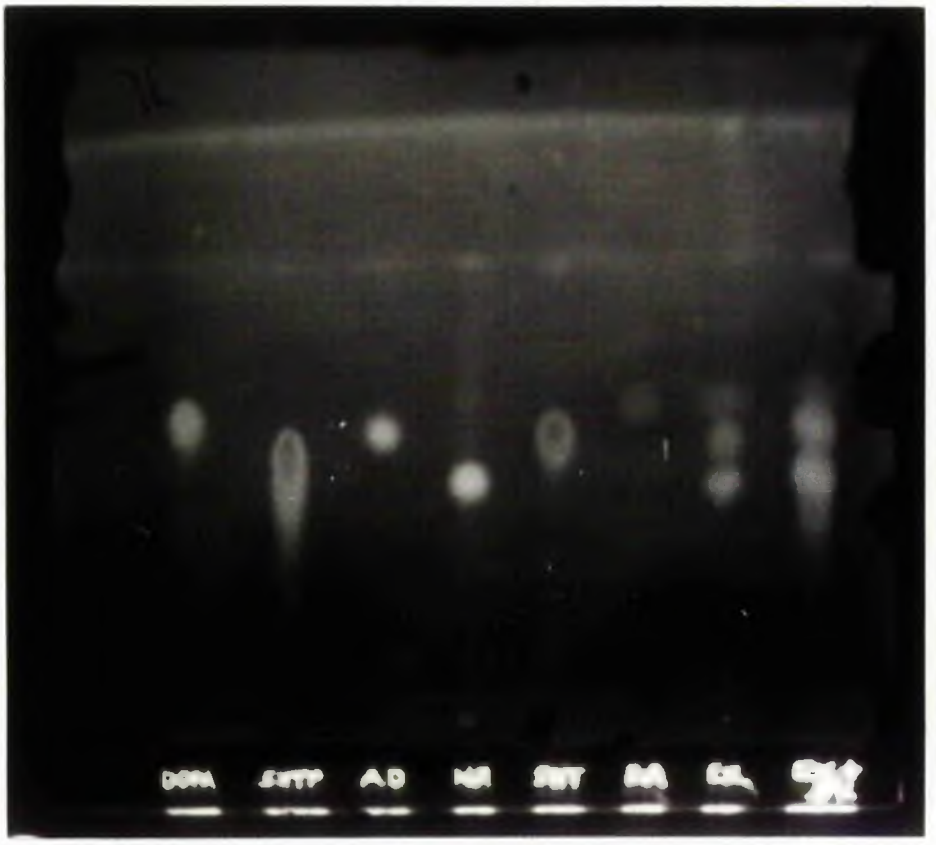

DOPA
5HTP
AD
NA
5HT
DA
DA

Table 2. Identification of amines on developed chromatograms (by method of Bell and Somerville 1966), based on Rf values and coloration of fluorescence in different solvent systems.

Table 3. Summary of different concentrations of amine in heart and brain tissues.

TABLE 2

COMPOUND	Paper Chromatography Phenol/HCl/KCN	Butanol/HCl	Thin layer chromatography Butanol/HCl	Fluorescent characteristic of amine after glycine-formaldehyde treatment.
DA	0.44	0.45	0.50	deep green
DOPA	0.41	0.32	0.49	pale green
NA	0.35	0.35	0.36	yellow green
AD	0.54	0.34	0.44	light green
5-HT	0.61	0.46	0.48	Yellow brown
5-HTP	0.55	0.32	0.41	Yellow

TABLE 3

	BRAIN µg/g wet weight	HEART µg/g wet weight	BRAIN:HEART
DA estimated chromato-graphically	3-6	0.4-0.8	15:2
5-HT estimated chromato-graphically	2-4	0.2-0.4	10:1
NA estimated pharma-cologically	0.06-0.1	0.8-1.2	2:25

	AURICLE µg/g wet weight	VENTRICLE µg/g wet weight	AURICLE: VENTRICLE
5-HT estimated Spectrophoto-metrically	0.29	0.15	2:1

If it is assumed that about 50% of each amine was recovered from heart and brain tissue after extraction and chromatography, then the comparison of the fluorescence intensities of spots with those of pure substances indicates the brain to contain 2-4 µg/g DA and 0.8-1.2µg/ NA.

(2) Estimation of NA.

Separate heart and brain extracts containing similar amounts of tissue were fractionated chromatographically, using butanol/HCl solvent, and the chromatograms subsequently dried. Areas of the chromatograms, corresponding to the Rf value of NA were eluted with a known volume of Krebs' solution containing ascorbic acid, and the eluates tested on isolated strips of rat stomach (Armitage and Vane 1964). Eluates from both heart and brain chromatograms caused the muscle to relax in a manner which was indistinguishable from that produced with pure NA. The degree of relaxation produced by the different extracts indicated that a greater amount of NA is present in the heart than in the brain (Fig 9a).

Crude extracts of similar amounts of heart and brain tissues, not separated chromatographically

were also assayed for NA content (fig 9b). The NA response was antagonised with propranolol.

It was calculated from the pharmacological results obtained that the brain contained about 0.08 µg/g NA and the heart 1 µg/g NA. The value obtained for heart tissue is similar to that calculated from chromatography.

(3) Estimation of 5-HT in auricle and ventricle.

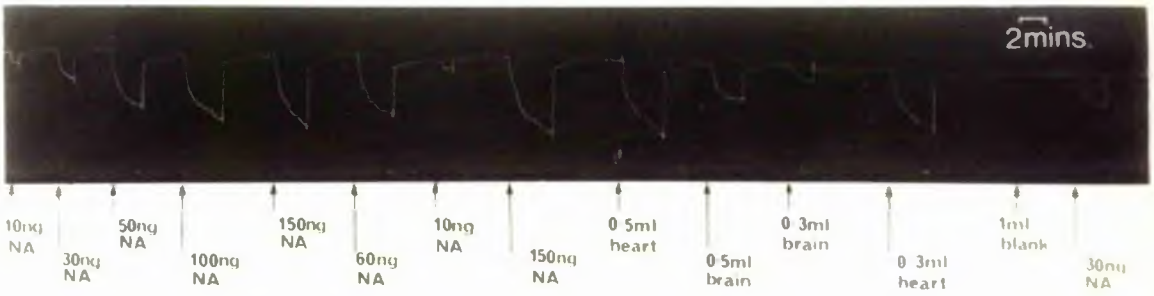
A calibration curve for 5-HT concentration (0.1 to 1.5 µg/ml) plotted against fluorescence was found to be linear (see fig 10). 5-HT was similarly recovered in a linear manner when added to the tissue. Initially the recovery was low (30-40%) but this was increased to about 65% by improvements in the method, such as using deionised water. Standard 5-HT and blanks were always prepared with the extracts, and the recovery for amine corrected.

The fluorescence spectra of purified auricle and ventricle extracts are shown in fig 11. When peaks of extracts are compared with standard concentrations of 5-HT (for ten determinations), the concentration of amine in the ventricle is calculated to be 0.15µg/g and the auricle 0.29µg/g.

Fig 9. (a) Effects of different concentrations of NA and some eluates from paper chromatograms of heart and brain extracts of Helix pomatia on the isolated stomach strip preparation. The samples marked heart and brain were taken from areas of chromatograms corresponding to the Rf value of NA. The blank sample was prepared from a part of the chromatogram close to the solvent front. Each segment of paper was eluted with 1.5 ml of Krebs' solution. In this particular experiment, the Krebs' solution contained 1µg 5-HT / L of saline.

(b) Effects of crude extracts of heart and brain and noradrenaline on the stomach preparation. The Krebs' solution contained 15 µg/L of 5-HT, DA and acetylcholine. Each extract contained tissue from twelve animals in 1 ml of saline.

9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0·3ml heart
1ml blank
30ng NA

B

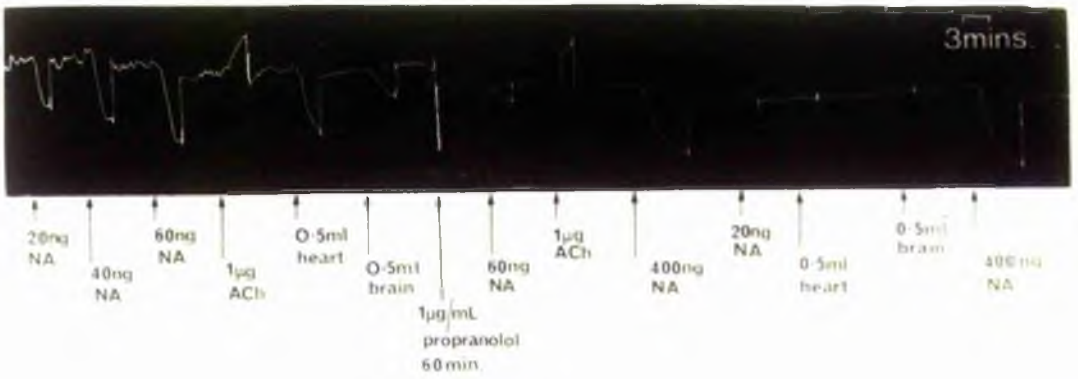
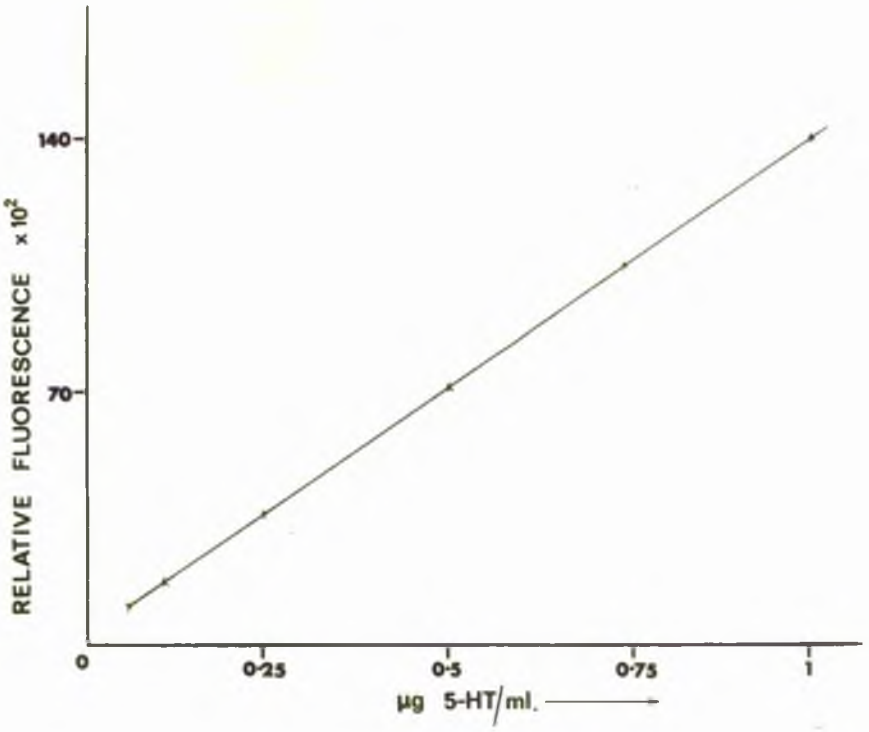

3mins
20ng NA
40ng NA
60ng NA
1µg ACh
0·5ml heart
0·5ml brain
1µg/mL propranolol 60 min
60ng NA
1µg ACh
400ng NA
20ng NA
0·5ml heart
0·5ml brain
400ng NA

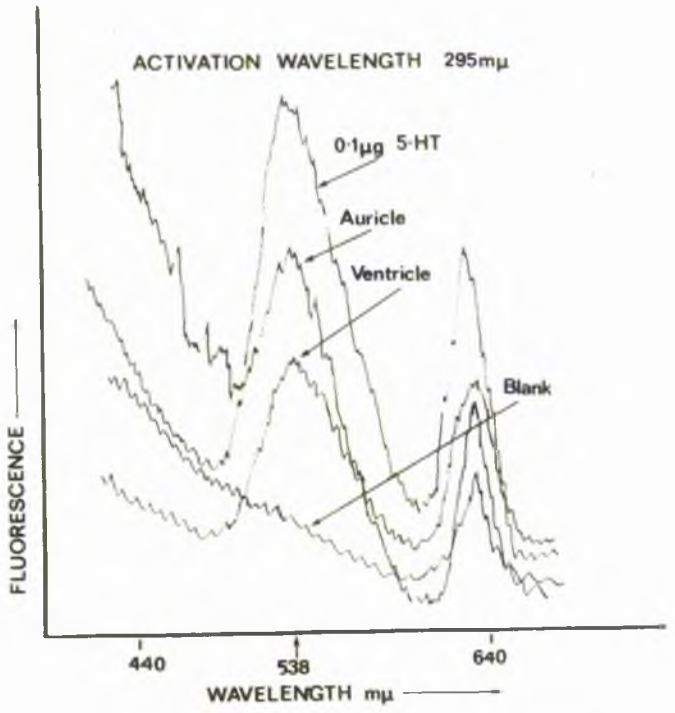
Fig 10. Standard calibration curve for 5-HT relating fluorescence intensity with amine concentration.

Fig 11. Fluorescence spectra of auricle and ventricle extracts prepared for detecting 5-HT.

10


RELATIVE FLUORESCENCE x 10²
140
70
0
0·25
0·5
0·75
1
µg 5-HT/ml.

11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0·1µg 5-HT
Auricle
Ventricle
Blank
FLUORESCENCE
440
538
640
WAVELENGTH mµ

DISCUSSION AND CONCLUSIONS

The amount of DA (3–6 μg/g) in the brain of *Helix pomatia*, as estimated by chromatography is in agreement with previous results. Cardot (1963); and Cardot and Ripplinger (1963) using paper chromatography and colorimetry calculated that the brain of *H. pomatia* contained 2–4 μg/g DA. This was similar (7–25 μg/g DA) to the content shown spectrophotofluorometrically by Dahl, Falck, Lindqvist and Mecklenburg (1962), and by Dahl, Falck, Mecklenburg, Myhrberg and Rosengren (1966) to occur in the cerebral ganglia of *H. pomatia*. The content of DA (5.5 μg/g) in the brain of *H. aspersa* is of the same order (Kerkut, Sedden and Walker 1966).

The concentration of 5–HT in the brain (2–4μg/g) also agrees well with that estimated by spectrophotofluorometry by Dahl, Falck, Lindqvist and Mecklenburg (1962), and by Dahl, Falck, Mecklenburg, Myhrberg and Rosengren (1966) in *H. pomatia* (3.77 μg/g 5–HT,) and calculated by Kerkut and Cottrell (1963) in *H. aspersa* (0.5–4 μg/g 5–HT). Similarly the amount of 5–HT in the heart (0.2–0.4 μg/g) is of the same value (0.2–0.5μg/g 5–HT) estimated to occur in heart tissue of molluscs (Welsh and Moorhead 1960), using spectrophotofluorometry.

NA was thought to be absent from tissue of gastropod molluscs (page 14). The method I used (see Armitage and Vane 1964) is most sensitive to NA; as little as 10 ng of amine produced a relaxation of stomach muscle. The results show the occurrence of small amounts of amine in heart and brain extracts, as indicated by the presence of its metabolites (see page 68). The low concentration of NA (0.08 µg/g) in brain tissue may not directly reflect a proportionately less important functional role for NA compared with DA (3-6 µg/g) in the central nervous system. It is possible that the low level of amine represents a higher rate of turnover than that of DA. However, the relatively smaller quantities of NA breakdown products in the brain suggest that the rate of turnover of NA cannot be so much greater than that of DA.

The function of NA (0.8-1.2 µg/g) and DA (0.4-0.8 µg/g) in the Helix heart is not clear. Both substances occur in cardiac nerves which presumably innervate the heart musculature (see page 100). Furthermore, both substances influence the mechanical activity of the isolated heart (see fig 47, and for explanation, page 125), but only in high concentrations

when compared with 5-HT, the presumed cardio-excitatory transmitter (see page 49).

The occurrence of about twice the concentration of 5-HT in the thin walled auricle (0.29 µg/g 5-HT) as compared with the muscular ventricle (0.15 µg/g 5-HT) argues for a specialised localisation of the amine, rather than a uniform distribution. This observation would seem to conflict with that of Rózsa and Za-Nagy (1967), who interpret their results as indicating that 5-HT is localised in muscle cells of the heart.

SUMMARY

A summary of the above results is shown in Table 3.

THE INTRINSIC INNERVATION OF HELIX HEART

INTRODUCTION

Welsh in 1953 was one of the first to suggest a physiological function for 5-HT in an invertebrate. Since 1953, various workers have obtained experimental evidence which supports a transmitter role for the amine in the molluscan heart (see page 49). However, although 5-HT has been detected in neurons of gastropod ganglia, its presence in nerve fibres of the heart has not yet been demonstrated. Since it has been suggested that the amine is localised in muscle cells of the heart (Rózsa and Zs-Nagy 1967), it was important first to describe the innervation of nerves within the heart, and then to proceed to study the localisation of 5-HT. My aim therefore was initially to discover the pattern of intrinsic innervation of the heart.

The extrinsic nerve supply to the Helix pomatia heart was described by Ripplinger (1957), as consisting of two branches which originate in the visceral nerve. One branch enters the heart at the junction of the pulmonary vein and the auricle and the other joins the heart at the apex of the ventricle at the point where it connects with the aorta.(fig 12).

Fig 12. Schematic diagram of *Helix* heart to show the extrinsic innervation. It indicates the dense accumulation of nerve fibres, proposed to have a neurosecretory function.

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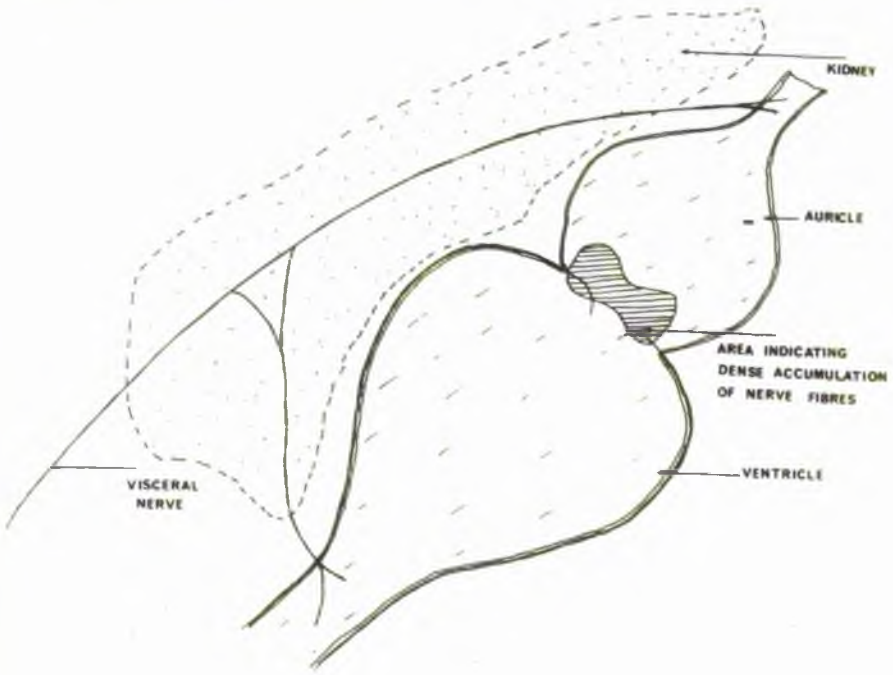
KIDNEY
AURICLE
AREA INDICATING
DENSE ACCUMULATION
OF NERVE FIBRES
VENTRICLE
VISCERAL
NERVE

Although Ripplinger proposed a network of nerve fibres within the heart from the results of physiological experiments, he did not observe the distribution of nerve fibres within the heart itself.

METHODS

1. Silver method (a slight modification of the method described by Fraser Rowell (1963).

Heart tissue was fixed in Carnoy solution embedded in paraffin wax, and sections cut from 8-20µ in thickness.

The following schedule was as follows:

(a) Sections dewaxed in xylol and brought down through the alcohols to water.

(b) Transferred to 20% silver nitrate in the dark for 1 hr.

(c) Rinsed in distilled water.

(d) Incubated at 30°C to 70°C for 16 hrs in:

0.2M boric acid)
0.05M borax) to give a pH 7.4, 40ml.

1% silver nitrate, 20 ml.

2,6 lutidine (dimethyl pyridine) 10 ml

distilled water, 250 ml.

(e) Washed well in distilled water.

(f) Transferred to 2% sodium sulphite (crystalline). 2 mins.

(g) Washed well in distilled water.

(h) Developed at 20°C for about 5-10 mins in:

5% silver nitrate, 9 ml.

9% sodium sulphite (crystalline) 300 ml.

0.5% hydroquinone, 20 ml.

(i) Rinsed in distilled water.

(j) Toned in 0.2% gold chloride solution, acidified with a little acetic acid, 5 mins

(k) Rinsed quickly in distilled water.

(l) Reduced in 2% oxalic acid, 2 mins.

(m) Rinsed in distilled water quickly.

(n) Fixed in 5% sodium thiosulphate, 3 mins.

(o) Washed in running tap water, 10 mins.

(p) Dehydrated in graded alcohol series and mounted.

2. Methylene blue staining (a modification of the method described by Pantin, 1962).

The staining solution was prepared as follows: 0.4g of Rongalite (Gurr) and five drops of concentrated NCl were added to 10ml of 2% methylene blue solution. The mixture was heated until yellow in colour, filtered and then diluted five times with Meng's saline. Freshly prepared solution (0.5 ml) was injected into the peri-visceral haemocoel of the animal. Two hrs later, the heart

together with some surrounding tissues were dissected from the snail and fixed at 4°C in 10% ammonium molybdate, prepared in Meng's saline, for 24 hrs. During this period, the ammonium molybdate solution was changed five or six times. The preparation was washed in distilled water for 2 hrs dehydrated in graded alcohol series and mounted in D.P.X. (B.D.H.). In some instances, after dehydration, sections of heart were hand cut with a razor blade and then mounted.

3. Electron microscopy

Small pieces of heart tissues were fixed in cold 1% osmium tetroxide in 0.2M cacodylate buffer (final pH 7.2) for one hr, dehydrated in graded acetone series and embedded in Araldite. Silver to gold sections were cut out on an L.K.B. ultra microtome, mounted on uncoated copper grids, and examined in an A.E.I. E.M.6B electron microscope. Sections were stained on the grids with lead citrate (Reynolds, 1963) and 2% uranyl acetate.

RESULTS

The silver method revealed nerve fibres in amongst, and presumably innervating, the muscle fibres of both auricle and ventricle (fig 13). However, since the muscle fibres also took up the stain, it was often

very difficult to differentiate the nerves from surrounding tissues with this technique.

The methylene blue method proved more successful. Fine nerve fibres were observed throughout the auricle and ventricle. Generally the fibres were only sparsely distributed (fig 14). However, in the auricle side of the auricular-ventricular junction there was a profuse network of fibres (fig 15). As far as I could discern, these fibres appeared to end blindly in this zone. Pronounced varicosities could easily be seen along their lengths and sometimes very large swellings, which suggests the presence of cell bodies as shown in fig 15e.

Electron microscopical examination showed the occurrence of nerve fibres in all parts of the heart, but none was observed in the pulmonary vein and aorta. Most of the nerve endings contained granular vesicles (i.e. vesicles which contain an electron dense granule, see fig 16) but few agranular vesicles were present, an observation also recorded in the heart of Archachatina (Nisbet and Plummer 1968). Often different axons, in a single nerve fibre, contained granular vesicles with different dimensions (fig 17). Numerous electron dense granules in axons were particularly abundant in that part of the auricle which adjoined the ventricle. In

Fig 13. Section of the auricle of Helix pomatia stained with silver to show nerve fibres (The bar represents 100μ).

Fig 14. Another section of auricle tissue stained with methylene blue to show nerve fibres.
(The bar represents 100μ).

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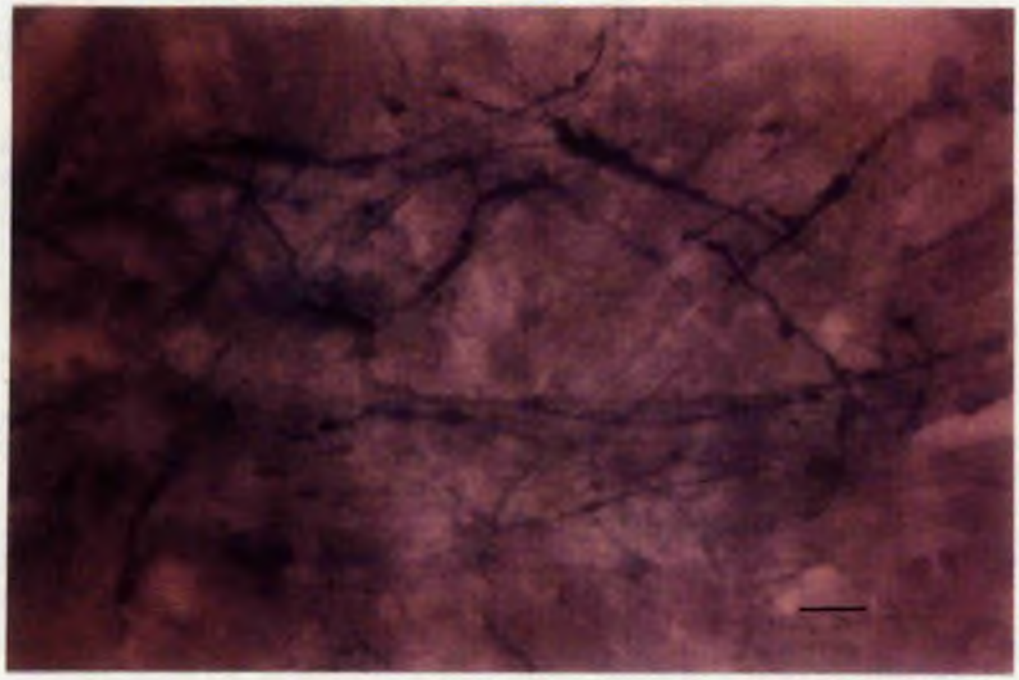

Fig 15. Appearance of nerve fibres at, or close to, the auricle-ventricular junction of the Helix heart in methylene blue preparations. (a) Numerous large swollen axons, or groups of axons, on the auricle side of the auricular-ventricular junction. The relative position of the ventricle (V) is indicated. (d) Higher power micrograph of part of the same area. (b) A similar area of heart to (a) in another preparation (c) Another example of the varicose appearance of fibres in the area. (e) A very large swelling, which may be a cell body, is indicated with the arrow.

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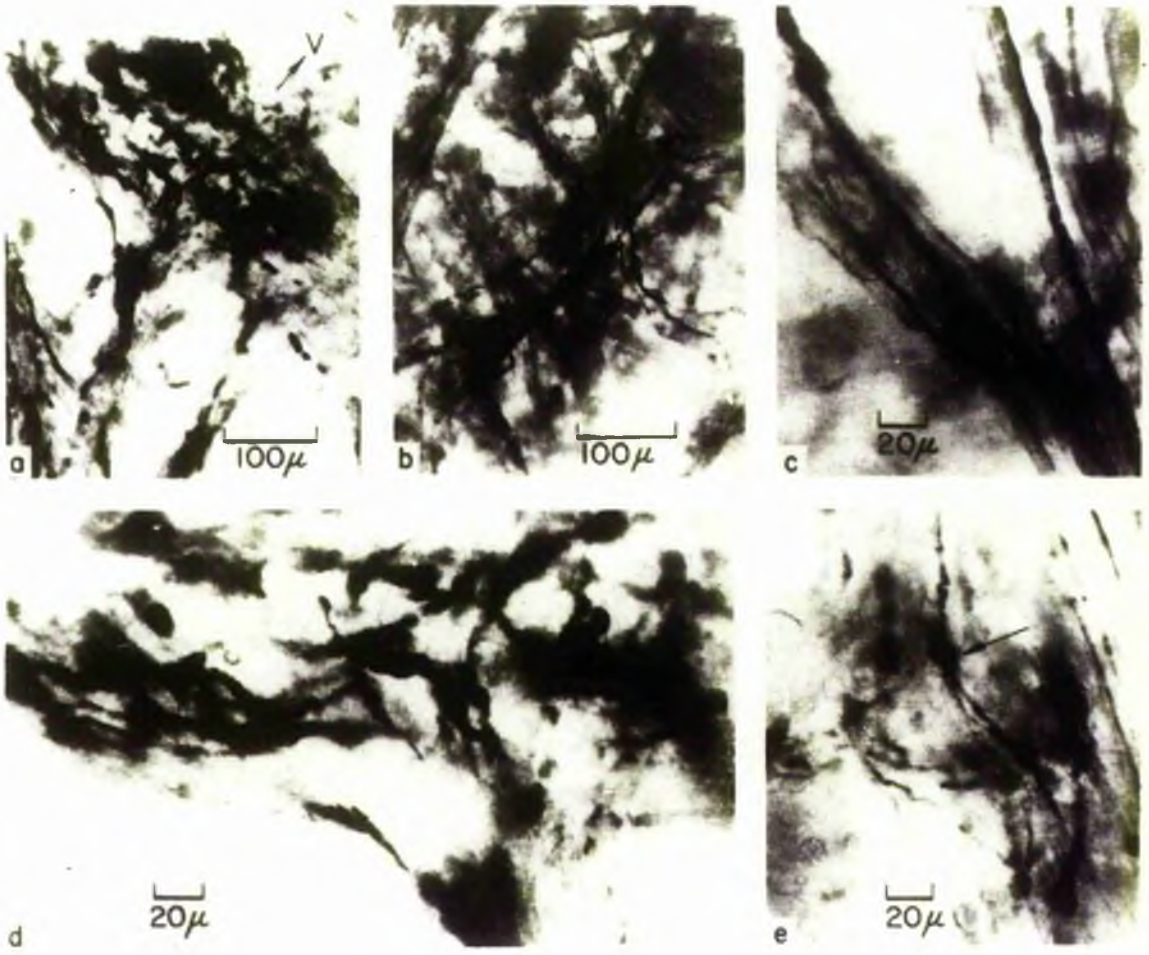

Fig 16. Section through the auricular-ventricular junction of heart of Helix. Most of the nerve fibres contain electron dense granules (The bar represents 1μ).

Fig 17. Different axons, in a single nerve fibre, containing granules of different dimensions (The bar represents 1μ).

Fig 18. Axons, containing large numbers of electron dense granules, close to or bordering on, the heart lumen (L) (The bar represents 1μ).

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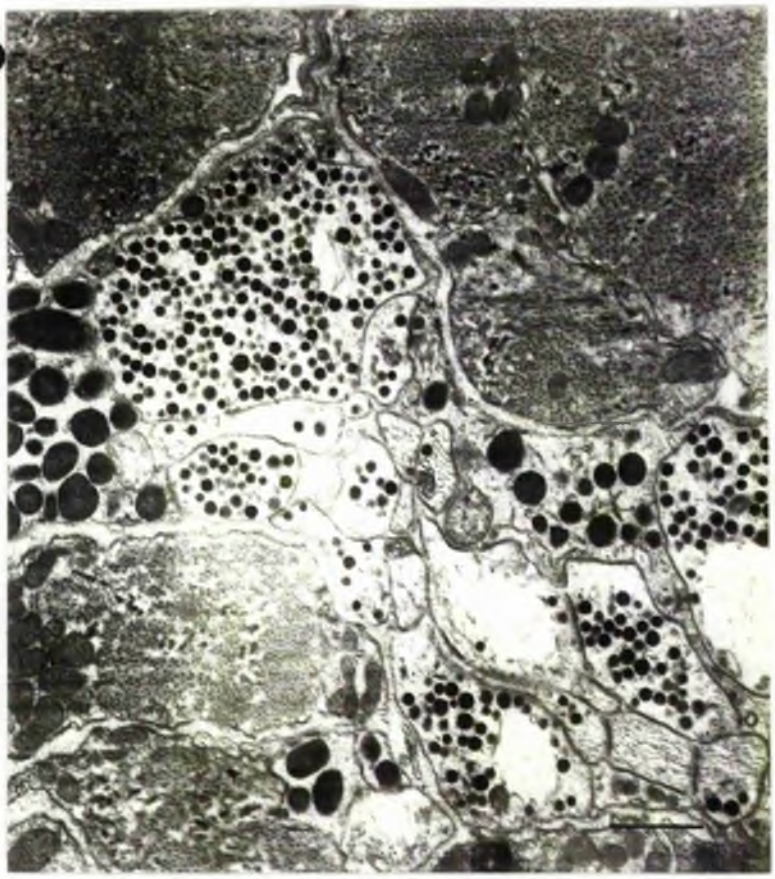

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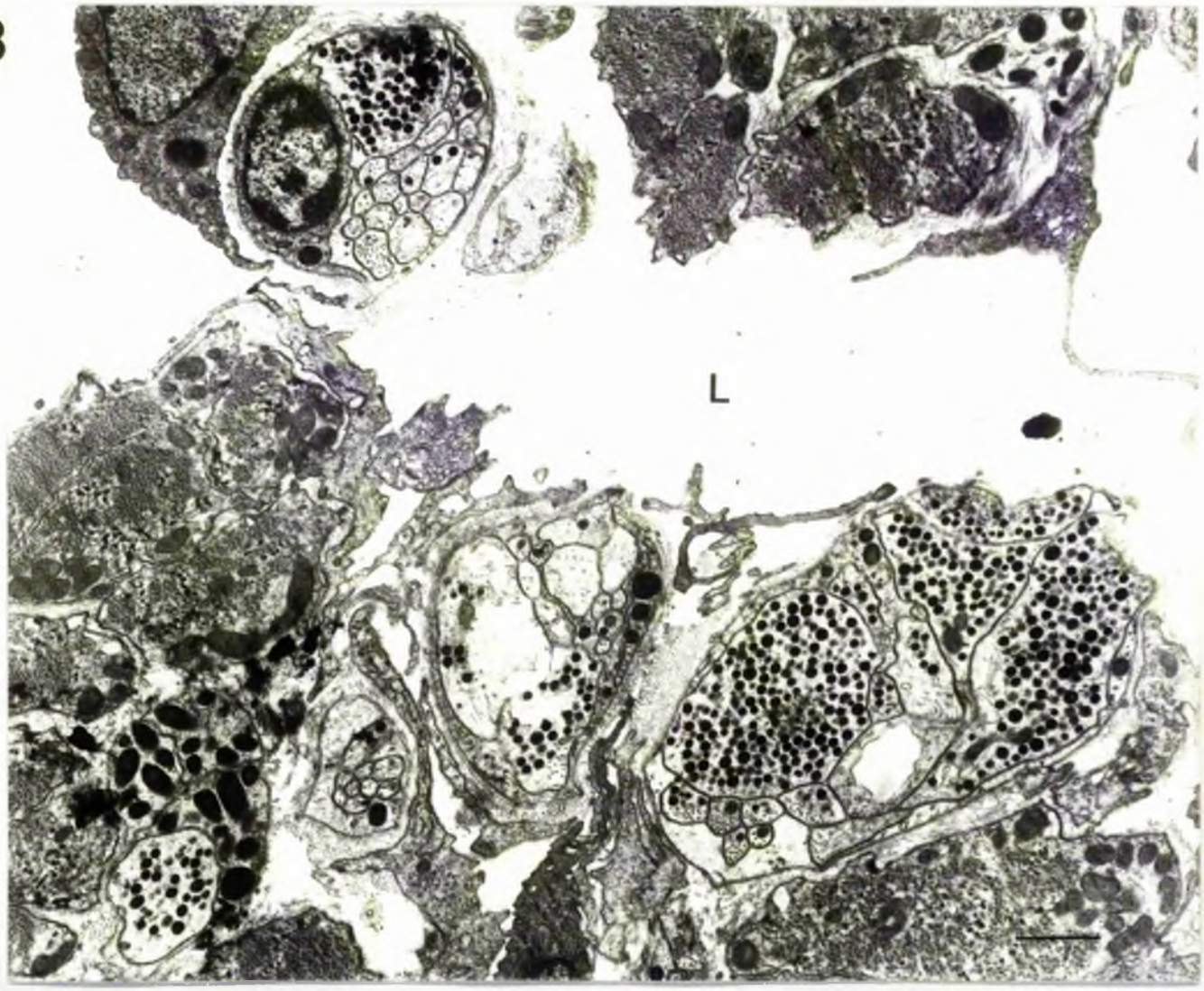


this area the axons were frequently seen to be very close or adjacent to the heart lumen (fig 18). Unlike the granules in axons in other parts of the heart, (size 80-150nm) these were slightly larger and measured from 100-250nm in diameter.

DISCUSSION

The overall distribution of nerves in the Helix heart is shown diagrammatically in fig 12. It came to my notice on completion of this work that Bey (1967) described a similar distribution of nerves in the snail heart (she did not comment upon the possible role of the nerves.)

The appearance and distribution of nerves in the auricular-ventricular area suggested to me that the system might have a neurosecretory function, liberating material directly into the lumen of the heart. The precise observations leading me to this conclusion were as follows:

(1) There was a relatively large number of nerve fibres in this particular region, compared with the comparatively scarce distribution in the rest of the heart.

(2) The fibres in this area appeared larger than those elsewhere in the heart and had pronounced swellings along their length.

(3) They appeared to end blindly at, or close to, the auricular-ventricular junction.

(4) The system appeared to stain more intensely when the methylene blue was passed through the centre of the heart (after injection into the perivisceral haemocoel) than when preparations were stained by injecting the same dye solution into the pericardial cavity. Thus most of the nerve fibres were probably situated close to, or bordering on, the heart lumen.

(5) Electron dense granules in the axons were larger than those in axons elsewhere in the heart, and were similar in appearance to the granules observed in known neurosecretory systems e.g. the neurohypophysial (Gerschenfeld, Tramezzani and De Robertis 1960).

Cottrell (see Cottrell and Osborne 1969a and Cottrell and Osborne 1969b) investigated further the function of the nerve fibres in the auricular-ventricular junction, using more sophisticated electron microscopic methods. He was able to show that:

(1) Tissue fixed with glutaraldehyde showed the presence of electron dense granules in axons situated in the auricular-ventricular junction of the heart, but not in other areas of the heart.

(2) Heart tissue processed by a modification of the chromaffin method (see Wood 1966, 1967) for the localisation of monoamines showed the occurrence of electron dense granule in axons situated in all areas of the heart.

(3) Tissues from animals pretreated with reserpine and then processed to localise monoamines revealed an absence of electron dense granules in axons in all areas of the heart, except those situated in the auricular-ventricular region.

On the basis of the observations described above it is suggested that the network of nerves in the auricular-ventricular area has a neurosecretory function whilst the sparse distribution of nerves in other areas of the heart (some of which contain monoamines) is involved in other functions.

The neurosecretory system appears to be analogous, though on a smaller scale, to the neurosecretory system of the vena cava of the cephalopod molluscs Eledone

(Alexandrowicz 1964) and Octopus (Alexandrowicz 1965). Granules with the same appearance have been seen in each situation (see Berry and Cottrell 1970, in Eledone) and (Martin 1968, in Octopus).

Theoretically, a neurosecretory system ending in the Helix heart is ideally situated for the release of an agent causing general stimulation of the animal. Assuming that the heart rate drops to a very low level during periods of inactivity (e.g. during hibernation), then the release of a "general stimulating substance" would first accelerate the heart and thus promote its own distribution around the rest of the animal. If the substance were released elsewhere under the same circumstances, it would take a longer period to be distributed and the initiation of stimulation would also take longer because the substance would not at first be able to increase the rate of circulation so effectively. Although such a function is feasible, the possibility of the existence of other or alternate functions cannot be excluded. It also seems likely that there could be a mechanical advantage in the release of

neurosecretory products from the nerve ending in, or close to, the auricular-ventricular junction of the heart, since observations of hearts beating *in situ* show that the junctional zone is greatly stretched by contractions of both the ventricle and auricle. Such movements might well aid the diffusion of material into the heart lumen by facilitating its release from the nerve endings.

CONCLUSIONS

The intrinsic innervation of the heart can be divided conveniently into two parts:

1) A neurosecretory system, situated on the auricle side of the auricular-ventricular junction.
2) A sparse distribution of nerves in all areas of the heart, but especially prominent at the junction of the pulmonary vein and the auricle and at the apex of the ventricle, at the point where it connects with the aorta.

FORMALDEHYDE HISTOCHEMICAL METHOD FOR THE LOCALISATION OF MONOAMINES

PRINCIPLES OF THE HISTOCHEMICAL PROCEDURE

The principles of the histochemical procedure developed by Falck 1962, will be briefly outlined. Pieces of tissues to be studied are dissected out as soon as possible after sacrifice, rapidly frozen in propane cooled in liquid nitrogen and dried in vacuo at -35°C. The biogenic monoamines are not destroyed by this procedure (Corrodi, Jonsson and Malmfors 1966) and will remain at their cellular sites in the freeze-dried tissues. The amines can then be converted to intensely fluorescent compounds by exposure of the tissue specimen to formaldehyde vapour (generated from standardised paraformaldehyde, Hamberger 1967) with such a low water content that no significant diffusion of the reactive amines and their reaction products occurs. After this step the specimen can be embedded in paraffin, sectioned, deparaffinised, mounted in non-fluorescent mounting medium (e.g. liquid paraffin) and examined in the fluorescent microscope. The histochemical procedure and the problems involved have been described by several authors (e.g. Dahlström and Fuxe 1964; Falck and Owman 1965; Corrodi and Jonsson 1967 and Ertfnkö 1967).

COMMENTS

1. Chemistry

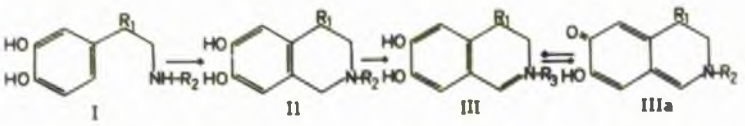
The histochemical fluorescence method is based on the following reactions: if amines are enclosed in dried protein, as in freeze-dried or air dried tissues, and treated with slightly humid paraformaldehyde gas, tetrahydro-derivatives are primarily formed (Piclet-Splengler reaction). In the solid state the tetrahydro-derivatives are immediately dehydrogenated to their corresponding fluorescent, 3,4-dihydrocompound in a protein promoted reaction (see Corrodi and Jonsson 1967). The fluorescent compounds formed in this way from catecholamines are 6,7-dihydroxy-3,4-dihydroisoquinolines (fig 19a) while that from 5-HT is 6-hydroxy-3,4-dihydro-β-carboline. (fig 19b). These two types of substances have their peak of emission at 480μ (appears green in the microscope) and 525μ (appears yellow in the microscope) respectively.

2. Specificity of the method and differentiation of monoamines

The fluorescence method in itself possesses a very high chemical specificity for catecholamines, 5-HT and their immediate precursors (DOPA and 5-HTP). It is however always necessary to examine whether the fluorescence observed is specific i.e. due to the presence of one of the reactive monoamines. This can safely be done by several

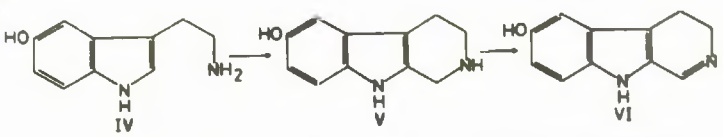
Fig 19. Histochemical reaction of (a) catecholamine with formaldehyde vapour to form 6,7-dihydroxy-3,4-dihydroisoquinolines, and (b) 5-hydroxytryptamine with formaldehyde vapour to form 6-hydroxy-3,4-dihydro-β-carboline.

a



The histochemical reaction between a catecholamine (I) and formaldehyde (dopamine R_1 = R_2 = H; R_3 = ; noradrenaline R_1 = OH; R_2 = H; R_3 = ; adrenaline R_1 = OH; R_2 = R_3 = CH_3).

b



The histochemical reaction between 5-hydroxytryptamine and formaldehyde.

histochemical tests, combined if possible with pharmacological tests. Factors which interfere in fluorescence microscopical analysis of monoamines are usually the general background fluorescence caused by tissue proteins and various autofluorescent structures which often include collagen and elastic fibres (see Sjöstrand 1944; Koenig 1963; Sainte-Marie 1965; Ritzen 1967a). Fluorescent tissue structures believed to be caused by monoamines were studied in relation to the following:

a) Reaction condition for development of fluorescence

The most important reaction variables are the humidity of the formaldehyde gas, the incubation time and the temperature. At a constant temperature of 80°C, exposure of freeze-dried tissue to formaldehyde gas converts monoamines into their 3,4-dihydro-compounds within 1 hour; to convert AD it takes three hours (Corrodi and Hillarp 1963; Falck, Häggendal and Owman 1963).

The reaction needs a certain amount of water in order to take place, and for practical purposes it has been found suitable to use the water absorbed into the paraformaldehyde as the main source (Hamberger,

Malmfors and Sachs 1965; Hamberger 1967). The amount of water absorbed into tha paraformaldehyde is very critical, as shown by several investigators, (see Malmfors 1965). If the formaldehyde gas used is too dry, the observed fluorescence will be weak and distinctly localised. With an increasing water content in the gas, the fluorescence intensity will gradually increase to an optimum, after which diffusion of the monoamines and/or their reactive products will occur. It was found for molluscan tissue that optimum fluorescence was obtained by exposing tissue to paraformaldehyde which had been stored at a relative humidity of 65%, at 80°C for 1 hour.

For quantitative determinations, it was therefore of great importance to carry out experiments in constant conditions viz:

1) Freeze-dry tissue for identical periods, so that the amount of water left in tissue is constant.
2) Use standardised paraformaldehyde (for gastropod tissue; best results were obtained with paraformaldehyde which had been stored for at least a week over sulphuric acid, density 1.27 g/litre to give a relative humidity of 65%).

3. Incubate freeze-dried tissue for 1 hour (at 80°C) with formaldehyde gas in a closed vessel.
4. Handle tissue before and after reaction with formaldehyde gas in such a way that water from the air does not affect it.

b) Spectral characteristics

The fluorophores of catecholamines and DOPA exhibit maximal excitation and emission at 410 and 480 mμ respectively, while those of 5-HT and 5-HTP have their peaks of excitation at 410 and emission at 525mμ. Under the fluorescence microscope fluorophores which release light at 480mμ appear green, and those which emit light at 525mμ are yellow in colour. However the colour the eye interprets not only often depends on the emission wavelength but also on the concentration of the fluorophore. For this reason, high concentrations of catecholamines can appear yellow (Caspersson, Hillarp and Ritzén 1966; and Ritzén 1967b). Microspectrophotofluorometry is required to overcome this difficulty, but unfortunately an instrument was not obtainable for the present investigations.

c) Sodium borohydride reduction

The specificity of monoamine fluorescence can be tested by treating tissue sections with low concentrations of sodium borohydride in isopropranol. During this treatment the fluorescent compounds of monoamines, 3,4-dihydroisoquinolines and β-carbolines are very rapidly and quantitatively reduced to their corresponding non-fluorescent tetrahydro-derivatives. These can then be converted to their fluorescent 3,4-dihydro-compounds by renewed exposure to formaldehyde gas (see Corrodi, and Jonsson, 1967). The non-specific fluorescence (e.g. auto-fluorescence and protein fluorescence) in tissue sections has not been found to undergo any typical changes on sodium boro-hydride treatment (Baumgarten, Holstein and Jonsson 1967). This is a simple and reliable test of high specificity; it must be remembered, however, that the actual proof of the specificity is the regeneration of the fluorescence after reduction, and sufficient controls were always treated parallel with the sections to be tested to exclude extraction of the fluorescent compounds. Solvent extraction is usually due to insufficient binding of the fluorophor; this, however can be reinforced by treating the tissue to be tested with formaldehyde gas of optimum humidity for 2-3 hours (see Corrodi and Jonsson, 1967).

d) Sensitivity to irradiation with ultraviolet light.

The formaldehyde-induced monoamine fluorescence is sensitive to irradiation with ultra-violet light, which results in a decreased fluorescence intensity (Jonsson 1967). Since the velocity of decomposition for 3,4-dihydro-β-carbolines is much greater than that for 3,4-dihydroisoquinolines, prolonged exposure to U.V. light can be used as a method to differentiate between the catecholamines and 5-HT.

e) Quenching of fluorescence in water.

Fluorescent products of monoamines exhibit a considerably stronger fluorescence in the solid state than in solution (Ritzen 1967b), and when enclosed in a dried protein matrix - as in model and tissue systems - they show a fairly weak fluorescence if surrounded by a water-containing medium (Falck 1962). The fluorescence intensity reappears after dehydration, provided the fluorescent compounds have not been accidentally extracted during the procedure. The quenching of the specific monoamine fluorescence by water is very specific.

f) Pharmacological analysis.

Biochemical and pharmacological investigations have shown that monoamine levels can be increased or

decreased in several ways, especially after administration of drugs interfering with monoamine metabolism (see page 100).

3) Sensitivity

Studies on both model systems and tissues have shown the fluorescence method to possess a very high sensitivity. In approximately 10μ thick protein layer (models), primary catecholamines and 5-HT can be demonstrated in concentrations of 0.0005 - 0.0001% (weight/volume). In practice, however, this method appears more sensitive for detection of catecholamines, probably because:

1) 5-HT does not react with formaldehyde as easily as the primary catecholamines (Corrodi and Jonsson 1965).
2) The fluorescent yield of 3,4-dihydro-β-carbolines is less than that of 3,4-dihydroisoquinolines (Jonsson 1967).
3) The fluorescent compound from 5-HT is very sensitive to irradiation with ultraviolet light.

Fuxe and Jonsson (1967) described a modified histochemical procedure which increased the sensitivity

for the demonstration of 5-HT. The method involves primary treatment of tissue with formaldehyde gas of a humidity which does not cause any diffusion. Then retreatment of the tissue specimens with a formaldehyde gas of high humidity results in increased yield of the final fluorescent 5-HT compound. This method was used successfully for localising 5-HT nerves in gastropod tissues, but unfortunately diffusion of any catecholamine products resulted simultaneously. Nevertheless, although limited, the method was found very useful in studying the distribution of monoamines in different tissues of the slug.

HISTOCHEMICAL LOCALISATION OF MONOAMINES IN THE HEART OF THE SNAIL HELIX POMATIA

INTRODUCTION

The results of earlier studies (see Table 3) show the occurrence of 5-HT, DA and NA in Helix heart tissue. Having also discovered the pattern of intrinsic innervation of the heart (see page 42), it was decided to study the localisation of the monoamines. Since only catecholamine-containing neurons have been shown to exist in gastropod heart tissue (Rozsa and Zs-Nagy 1967), it was important to make a histochemical study of the distribution of 5-HT, especially since the amine is thought to be the cardio-excitatory transmitter. Drugs which are known to influence the tissue-content of 5-HT and/or catecholamines were used to substantiate the localisation of the different amine-containing nerves.

Procedure for localising amines

Tissues to be examined were rapidly dissected, orientated on small pieces of moist filter paper and frozen in liquid propane (calor gas), precooled in liquid nitrogen. After rapidly removing excess propane with filter paper, the samples were placed on the pre-cooled (-60°C) stage of a Pearse freeze-dryer (Speedivac-

Pearse Tissue Dryer, Model 1). The tissues were dried at a pressure of 10^{-3} torr, in the presence of phosphorous pentoxide and at a temperature of -35°C for at least three days. After drying, control tissues were immediately infiltrated with melted paraffin-wax at a reduced pressure and embedded. Dried tissues to be examined for the distribution of monoamines were exposed to formaldehyde gas for 1 hour at 80°C. The vapour was obtained from paraformaldehyde which had been kept at a relative humidity of 65% (Hamberger, Malmfors and Sachs 1965). Tissues were subsequently embedded under reduced pressure in paraffin wax.

Whole mount preparations and 10µ sections were examined using a Leitz microscope fitted with a dark field condenser, a B.G. 12 excitation filter, a 530 m barrier filter and an HBO mercury vapour lamp. Black and white photographs were taken with Kodax Tri-X film (ASA 400). Exposure time was 4-6 minutes. Colour photographs were produced from transparency film (Agfa-Gefaert, for artificial light; ASA 50). Exposure time was 4-10 minutes.

Injection of drugs

Each of the following drugs was injected into the foot of different snails, Nialamide (Sigma), and

α-methyl-m-tyrosine (Koch-Light Ltd), p-chlorophenyl-alanine (Pfizer Group), DOPA (Koch-Light Ltd), 5-HTP (Ralph N. Emanuel Ltd), m-hydroxybenzoxamine or NSD 1024 (Smith and Nephew Research Ltd) and reserpine (Ciba). 2.5 mgs of each drug was dissolved in 0.5 ml 0.1N HCl, neutralised with 0.1N NaOH and made up to 3 mls with Mang's saline. 3 ml quantities of each drug was injected in 0.3 ml aliquots, at three hour intervals, over a period of thirty hours.

RESULTS

1. Normal Hearts

Microscopical analysis of heart tissue revealed fluorescent nerve fibres that appeared yellow and/or green in colour. All the criteria to test the specificity (sodium borohydride test, quenching with water, sensitivity to ultraviolet light and examination of tissue not sublimated with paraformaldehyde) indicated that the fluorescence in the fibres represent biogenic amines. The specific fluorescence was characteristic of monoamines : no further fluorescence developed after prolonged exposure of tissues to formaldehyde gas, which would demonstrate structures as containing a secondary catecholamine, such as AD. These findings agree with chromatographic analysis showing only the presence of DA, NA & 5HT. There was however some natural background fluorescence, and a few autofluorescent granules

were scattered throughout the heart.

The auricle was supplied with a rich monoaminergic innervation. The nerve terminals ran in all directions, not necessarily following the course of muscle fibres (fig 20). The ventricle was supplied with a moderate number of monoaminergic nerve terminals and the innervation was usually less conspicuous than in the auricle portion of the heart. Nerve fibres in the ventricle often followed the direction of individual fibres of trabeculae, in a way suggesting a true musculature innervation (fig 21). No monoaminergic cell bodies were found in any parts of the heart.

Although some of the fluorescent nerve fibres appeared either green or yellow in colour, the majority of neurons were yellow-green in coloration; this suggested a close association of primary catecholamines with 5-HT in the heart.

2. Drug treated Hearts

Hearts from animals treated with nialamide, a MAO inhibitor, resulted in nerve fibres emitting a brighter light, so that many of the delicate varicosities could be seen easily under low power magnification (fig 22).

Treatment of snails with NSD 1024, a DOPA decarboxylase inhibitor, resulted in a slight decrease in

Fig 20. Fluorescent nerve fibres in the auricle portion of the Helix heart. Most of the fibres are yellow-green (yg) in colouration but some appear distinctly yellow (y) or green (g). Yellow autofluorescent granules (s) are scattered throughout the heart tissue. (The bar represents 100μ).

Fig 21. Amine-containing fibres in the ventricle part of the heart. Yellow green colouration of fibres (arrows) clearly seen. (The bar represents 100μ).

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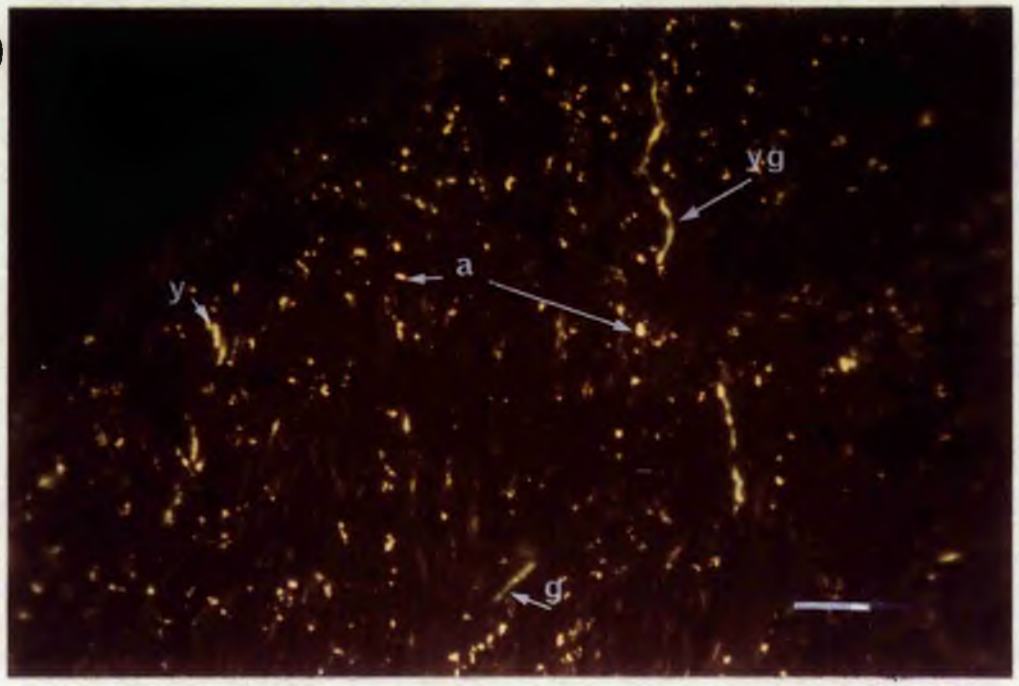

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Fig 22. Heart tissue from snail injected with nialamide. Amine-containing nerve fibres in auricle (a) and ventricle (b) are very intense. (The bar represents 100μ).

Fig 23. Heart tissue from snail injected with NSD 1024. Very few fluorescent nerve fibres could be discerned. (The bar represents 100μ).

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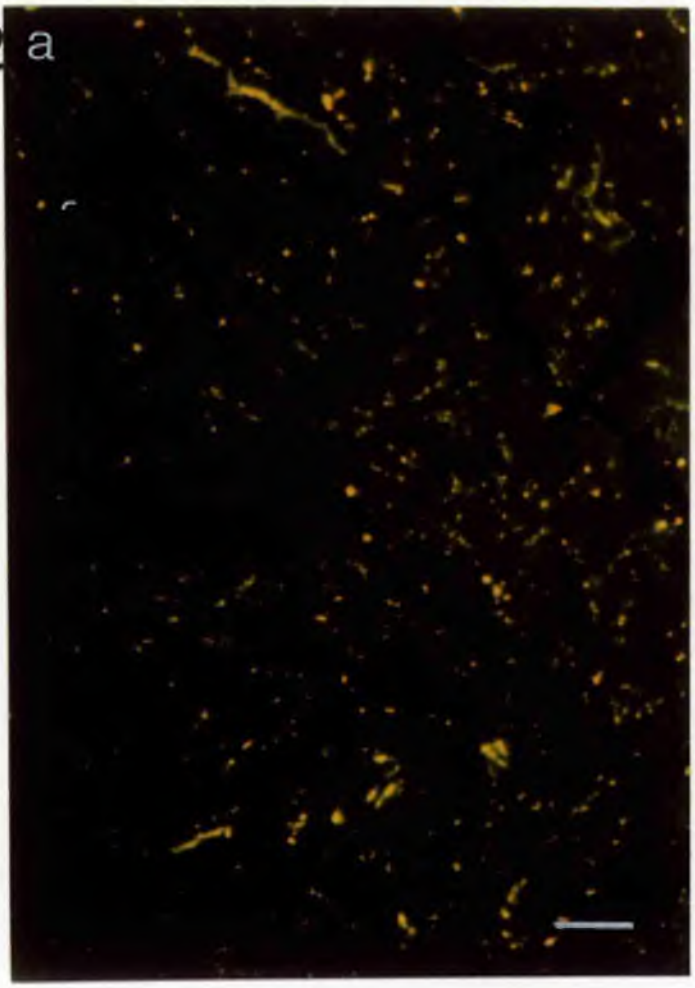

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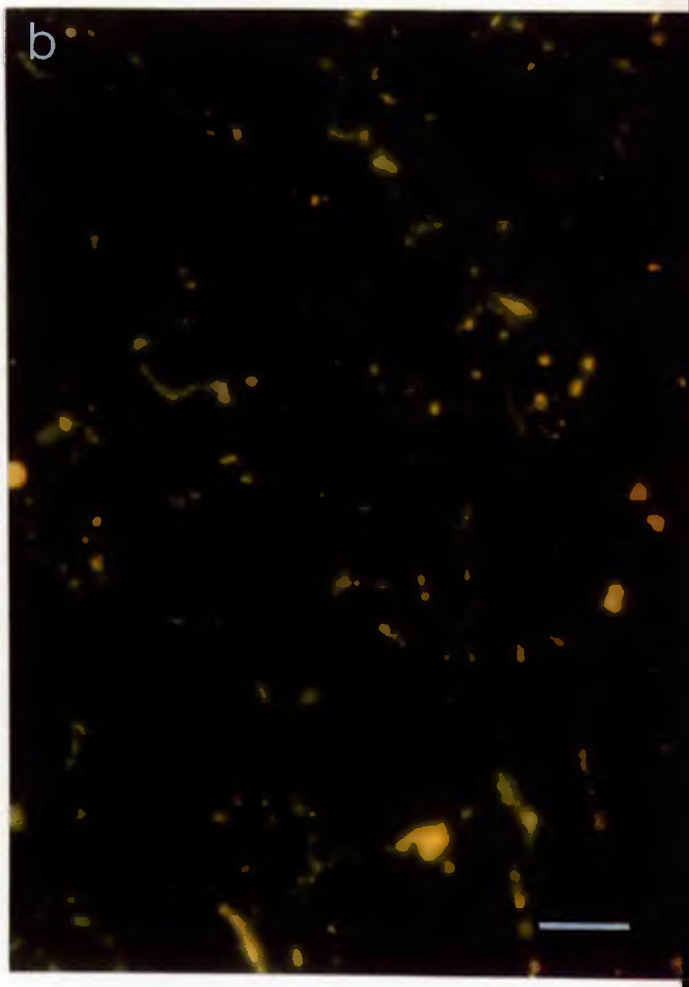

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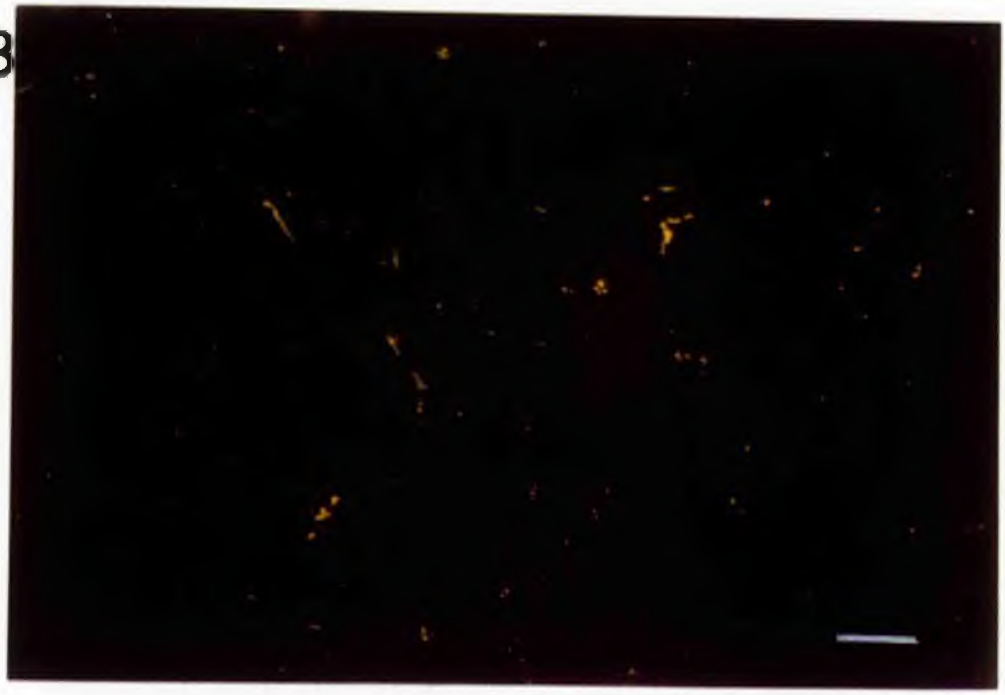

Fig 24. Heart tissue from animal injected with reserpine. Monoamine fluorescence completely eliminated. (The bar represents 100μ).

Fig 25. Heart tissue from snail injected with p-chlorophenylalanine. There is a decrease in the number of fluorescent fibres, the fibres that are present are more green in coloration. (The bar represents 100μ).

Fig 26. Heart tissue from snail injected with α-methyl-m-tyrosine. Again a decrease in amount of fluorescent fibres and those that remain appear yellow in colour. (The bar represents 100μ).

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Fig 27. Heart tissue from animal injected with 5-hydroxytryptophan. There is a great increase in the number of structures which appear yellow in colour. (The bar represents 100μ).

Fig 28. Heart tissue from snail injected with DOPA. All tissue appears dark yellow-green in colour under the fluorescence microscope. (The bar represents 100μ).

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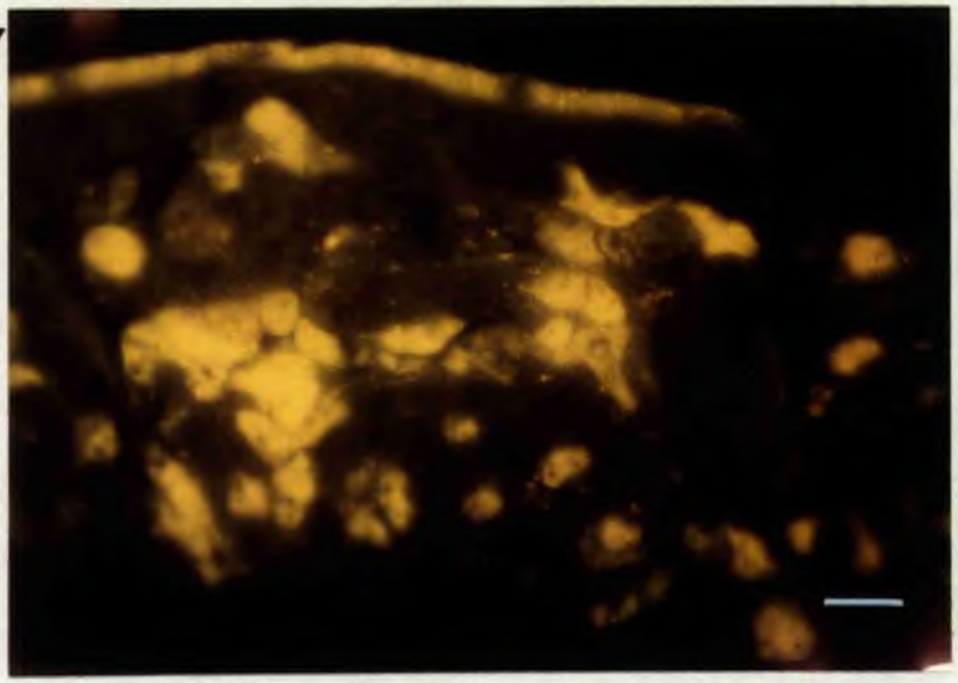

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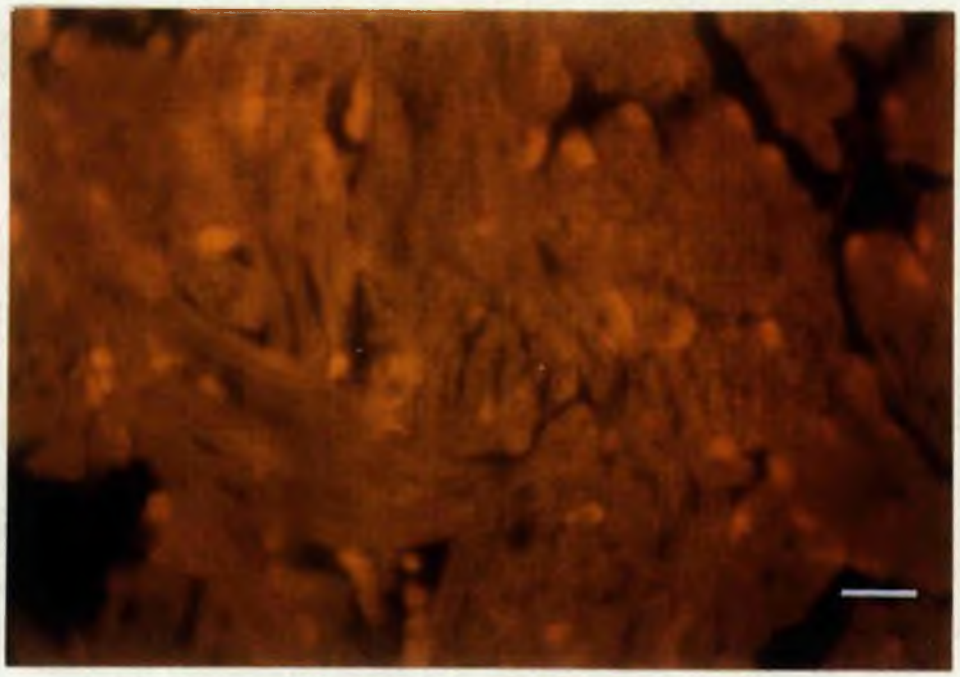

the intensity of formaldehyde-induced products in nerve fibres of the heart (fig 23).

Pretreatment of snails with reserpine, which depletes monoamine stores, dramatically reduced the specific fluorescence in the heart so that only a very few weak fluorescent varicosities could be seen. The background fluorescence was greater than in a normal heart tissue (fig 24).

Hearts from snails injected with p-chlorophenyl-alanine, which is known to deplete 5-HT stores in the vertebrates, showed a decrease in the amount of fluorescent fibres and varicosities, which when present reappeared green in colour (fig 25).

The drug α-methyl-m-tyrosine, is known to deplete catecholamine stores in the vertebrates, by inhibiting the enzyme tyrosine hydroxylase. Hearts from snails which had been injected with the drug showed a decrease in total fluorescent fibres, although the fibres which remained, especially prominent in the auricle, appeared yellow in colour (fig 26).

Fig 27 shows a section from heart of snail previously injected with 5-HTP, the precursor of 5-HT. There is a great increase in the number of fibres which

appear yellow in colour. In addition a number of bright yellow autofluorescent granules of unknown significance can be seen scattered throughout the musculature.

Pretreatment of snails with DOPA, (precursor of catecholamines) caused all the heart tissue to appear dark yellow-green when examined with the fluorescence microscope (fig 28). This made it impossible to observe any fluorescence in nerve fibres. However when the amount of DOPA injected was decreased to 0.2 mg, many green fibres could be seen, although the background tissue still emitted yellow-green light.

DISCUSSION

The intrinsic nervous system of Helix pomatia heart has been described (page 87). Specific monoamine fluorescence is associated with fibres and corresponds roughly to the sparse distribution of nerves. No fluorescence could be seen to match the dense network of fibres in the auricular-ventricular junction, proposed to have a neurosecretory function. Further, no evidence was obtained, even after injecting various drugs into snails, for the occurrence of monoamines in either muscle cells or nerve cell bodies.

On the basis of the nature of fluorescence in the heart, particularly after injecting drugs known to interfere with levels of individual monoamines, and of the result of chromatography (see Table 3), it is concluded that 5-HT, DA and NA are located in nerves within the heart. This deduction is further supported by Cottrell (see Cottrell and Osborne 1969b), who showed the presence of small amine-containing granules, which range in size from 80-130 nm in diameter, to occur in nerve endings of the heart.

Nerve fibres which appeared yellow-green in the fluorescence microscope could represent the localisation of concentrated amounts of catecholamines (see Caspersson, Hillarp and Rittén 1966 and Retzén 1967b). However, since only small amounts of DA and NA are present in the heart (0.4-0.8 µg/g DA and 1 µg/g NA) it seems unlikely. Pretreatment of snails with drugs which interfere with the metabolism of individual monoamines, shows that the yellow-green coloration represents both primary catecholamine and 5-HT. It is suggested that often different axons in a single nerve fibre contain either primary catecholamine or 5-HT, so that the overall picture of such a nerve fibre would

Table 4. Summary of the effects of various drugs on the fluorescence in the snail heart. 2.5 mg of each drug was administered over a period of 30 hours before observation.

TABLE 4

Name of Drug.	Effects	Effects upon fluorescence in snail heart
Reserpine	Depletes amines from molluscan nervous tissue (Mirroli & Welsh 1964)	Almost all fluorescence eliminated
p-Chloro-phenyl-alanine	Reduces 5-HT content by inhibiting the enzyme tryptophan hydroxylase in vertebrates (Koe & Weissman 1966)	Slight decrease total fluorescence. Increase in intensity of green fluorescence
α-Methyl-m-tyrosine	Reduces CA content by inhibiting the enzyme tyrosine hydroxylase in vertebrates (Moore 1966)	Slight decrease total fluorescence. Increase in intensity of yellow fluorescence
5-HTP	Precursor of 5-HT in molluscs (Welsh & Moorhead 1959)	Great increase of yellow fluorescence
DOPA	Precursor of CA's in molluscs (Carsot 1963)	Muscle cells fluorescent yellow-impossible to observe nerves
Nialamide	Monoamine oxidase inhibitor in vertebrates	Total fluorescence slightly potentiated
MSD 1024	DOPA decarboxylase inhibitor in molluscs (Perkut, Sedden & Walker, 1967).	Total fluorescence very slightly reduced

appear yellow-green in colour. This view receives support from electron micrographs of heart tissue which show transverse pictures of individual nerve fibres. In fig 17 a number of axons, each axon containing granular vesicles of different size, can be seen in a single nerve fibre. It could be argued that the only difference between the axons is that they contain granular vesicles at different stages of development, but it would appear more likely that each axon contains a single type of monoamine and that each amine is associated with a specific class of granular vesicles.

The localisation of 5-HT within nerve fibres of the heart is in agreement with the theory that the amine is a cardio-excitatory transmitter (see page 49) Its absence in muscle cells, as was indicated by spectrophotofluorometric results which showed a greater amount of 5-HT in the thin walled auricle compared with the muscular ventricle (see Table 3), does not favour the hypothesis put forward by Rosen and Zs-Nagy (1967), that 5-HT has an intracellular role within individual muscle fibres.

Finally the functional role of primary catecholamine-containing nerve fibres is not clear, especially since DA and NA only effect the activity of

the isolated Helix heart in high concentration compared with 5-HT (see Table 5).

CONCLUSIONS

1. Fluorescence microscopy of the heart of Helix pomatia shows a sparse distribution of monoamine-containing nerve fibres in the auricle and ventricle.

2. Pretreatment of snails with drugs which interfere with monoamine levels - summarised on Table 4 - shows 5-HT and primary catecholamines to be present in the fluorescent nerve fibres.

3. There was no evidence for the localisation of monoamines in the muscle cells of the heart.

HISTOCHEMICAL LOCALISATION OF MONOAMINES IN THE SLUG LIMAX MAXIMUS, AND THE DISCOVERY OF AN IDENTIFIABLE 5-HT GIANT NEURON

INTRODUCTION

Most of the neurons in the central nervous system of pulmonate and opisthobranch molluscs are very large, some reaching 0.8 mm in diameter. The cells are peripherally arranged on the surface of the brain immediately beneath the connective tissue, and it is easy to see the neurons and either to dissect specific cells from the brain or to insert a microelectrode into a selected neuron. Histochemical studies have shown that there are at least four types of cells in the gastropod brain: (1) those which do not contain monoamines, (2) catecholamine-containing cells, (3) 5-HT-containing cells, (4) cells containing both 5-HT and catecholamine (see page 23). It was decided to study the distribution of monoamine-containing neurons in the brain of Limax maximus in an attempt to locate a readily identifiable, monoamine-containing

giant neuron. The ultimate aim was to dissect the neuron from the brain and to investigate further the content and localisation of the amine within it.

The fluorescence histochemical technique was also used to study the occurrence of monoamine-containing neurons in other tissues of the slug for detailed information about the distribution of monoamines. The aim of this investigation was to compare the distribution of monoamines in the slug with other data available, in order to gain insight into the functional significance of primary catecholamines and 5-HT in peripheral tissue.

METHOD

The procedure described above for the localisation of amines in the heart of Helix was also adopted to study the distribution of monoamine-containing neurons in Limax maximus. To distinguish autofluorescence from fluorescence caused by monoamines, sections were immersed in 0.1% sodium borohydride in 98% isopropranol. This procedure reduces only the specific fluorescence, provided it is restored by exposure to formaldehyde gas (Corrodi,

Hillarp and Jonsson 1967). In addition, the influence of ultraviolet light on fluorescence was observed and sections of tissues not exposed to formaldehyde gas were examined for comparison with others not exposed.

In some experiments the level of amine fluorescence was increased by injecting nialamide. From dose-response experiments three doses of nialamide (300 mg/Kg body weight) in aliquots of 0.2 ml were injected into the pneumostomal aperture over a period of 10 hours. To deplete tissues of monoamines, 30 mg of reserpine per Kg body weight were injected five times in 0.2 ml quantities into the pneumostomal aperture of each animal.

RESULTS

1. Cerebral ganglia

Only a small proportion of neurons appeared green or yellow when viewed in the fluorescence microscope. Small green and some yellow cells were consistently observed in certain regions of the meso- and metacerebral portions of the ganglia. The central neuropile

regions of these areas showed intense green fibres and varicosities of different thicknesses. Most prominent, however, was a giant yellow neuron located on the ventral surface of the meta-cerebral part of each ganglion at the level of the origin of the lip nerves (fig 29). This cell is spheroidal in shape and is unipolar; the length across its major axis measures 180µ. As with other fluorescent cells, the cell nucleus did not fluoresce. A group of small (15-25µ) intensely fluorescing green cells was normally seen clustering around the edge of each of these cells nearest to the intercerebral commissure. The processes of the giant yellow cells were often inconspicuous, whereas axons of the green cells could be more easily observed. However, even the axons of the green cells, which could be traced to the neuropile, fluoresced less intensely than their cell bodies. The large collection of small neurons which forms the proto-cerebral ganglion did not fluoresce, although the ganglion's neuropile contained fluorescent green axons.

2. Suboesophageal ganglionic mass

The pleural, parietal and visceral ganglia are fused together and are situated dorsally to the pedal

Fig 29. Sections through the cerebral ganglia of Limax maximus, processed by the histochemical method to demonstrate monoamines. A pair of giant yellow neurons (large arrow heads) on the ventral surface of the meta-cerebral part of each ganglion is located at the level of the origin of the lip nerves (ln). This cell is unipolar in shape (29b) and the cytoplasm of the cell contains yellow granules (g). A group of small intensely fluorescing green cells (small arrow heads) are seen clustering around the edge of each of the yellow cells. Much os the neuropile region (n) contains green fluorescent fibres (In (29a) and (29b) the bar line represents 100μ. In (29c) the bar line represents 50μ).

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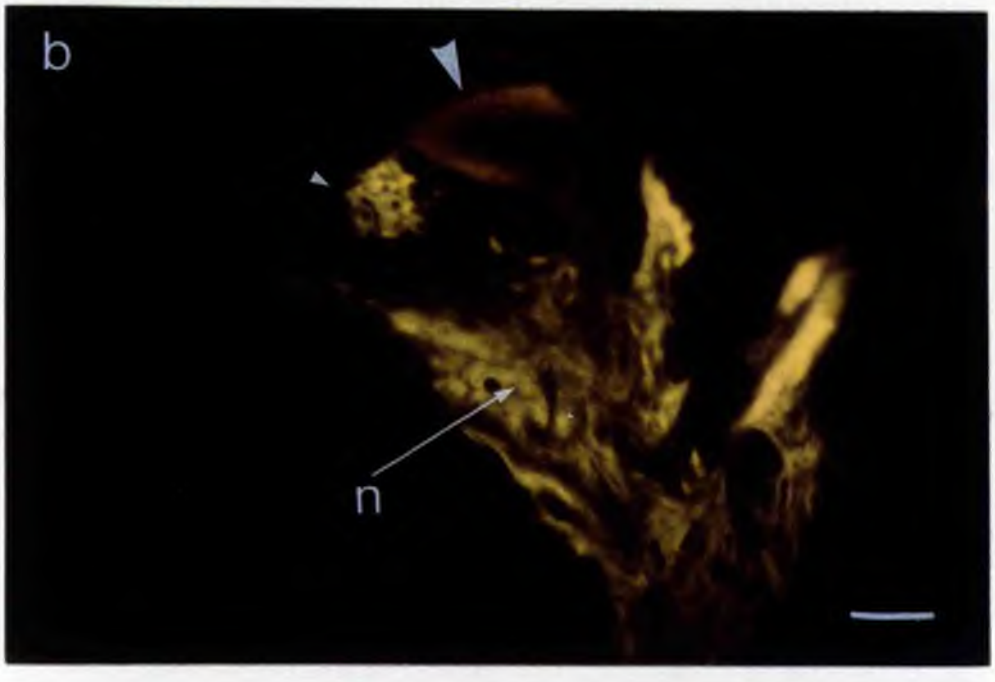

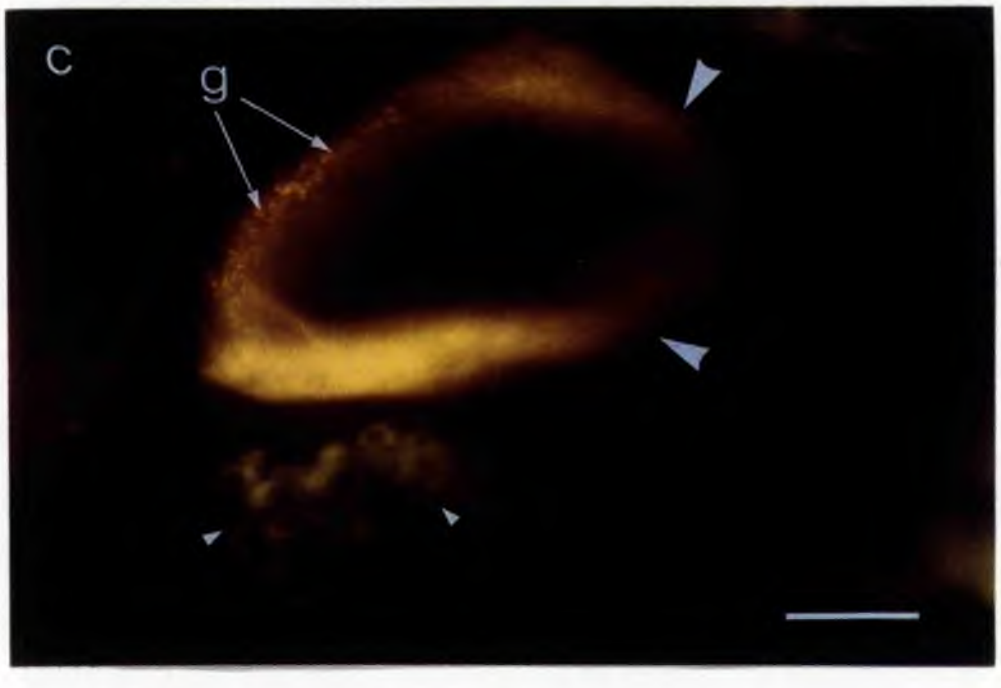

ganglion in the living animal. Together these ganglia make up the suboesophageal ganglionic mass. Many fluorescent neurons were observed in the different ganglia but even more in some, notably the visceral, right parietal and pedal ganglia. The fluorescent cells range in size from a pair of giant yellow neurons in the visceral ganglia of about 200μ in diameter (fig 30) to small green neurons of 15μ in diameter situated in large numbers on the medial-lateral borders of the pedal ganglia (fig 31). Although some green fluorescent cells range in size up to a maximum of 100μ in diameter as observed in the pedal ganglia, they were never as large as some giant yellow cells. Large numbers of yellow cells, 60μ in diameter were also seen in the ventral surface of each pedal ganglion. Generally the fluorescent neurons appeared either yellow or green in colour, but in some preparations certain neurons especially in the parietal ganglia, appeared yellow-green in colour. It would have been too time-consuming to carry out experiments to discover whether these cells contain a mixture of catecholamine and 5-HT.

Some of the central neuropile areas of the suboesophageal ganglionic mass showed a green fluorescence.

Fig 30. Section through the visceral ganglia, showing numerous varicosities. A pair of giant yellow neurons (arrows) can be seen at the edge of the ganglion. (The bar represents 100μ).

Fig 31. The pedal ganglion showing large numbers of fluorescent cells closely associated with many amine-containing fibres in the neuropile. (The bar represents 100μ).

Fig 32. Section through the pleural-pedal connective showing green fluorescent fibres in the wall of the statocyst. Many non-fluorescent cells can be seen associated with fluorescent varicose fibres in the neuropile. (The bar represents 100μ).

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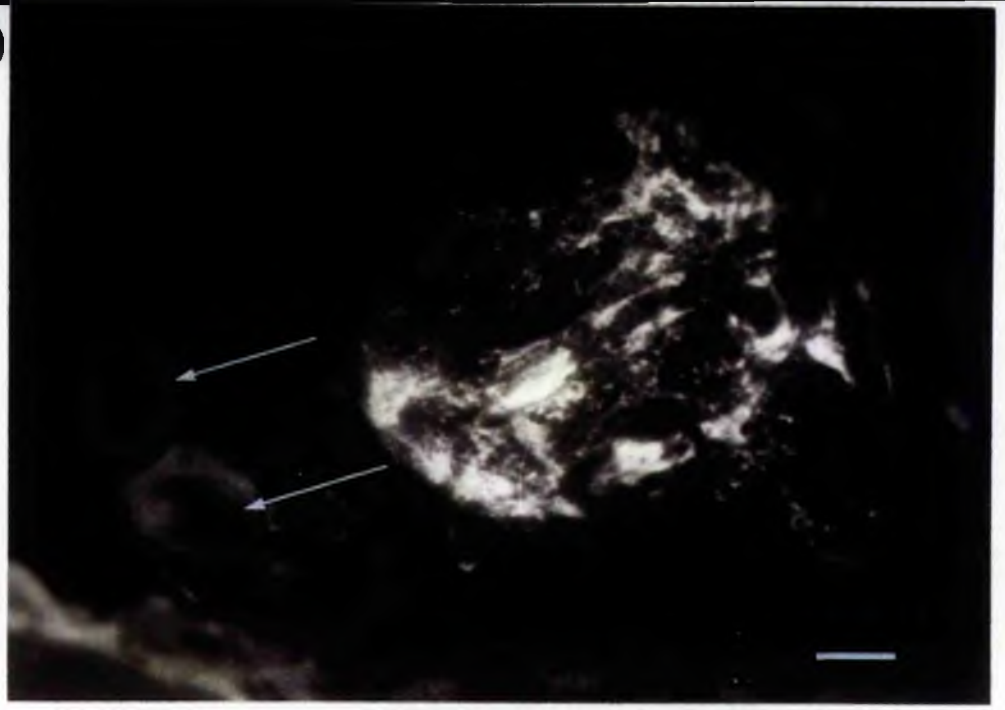

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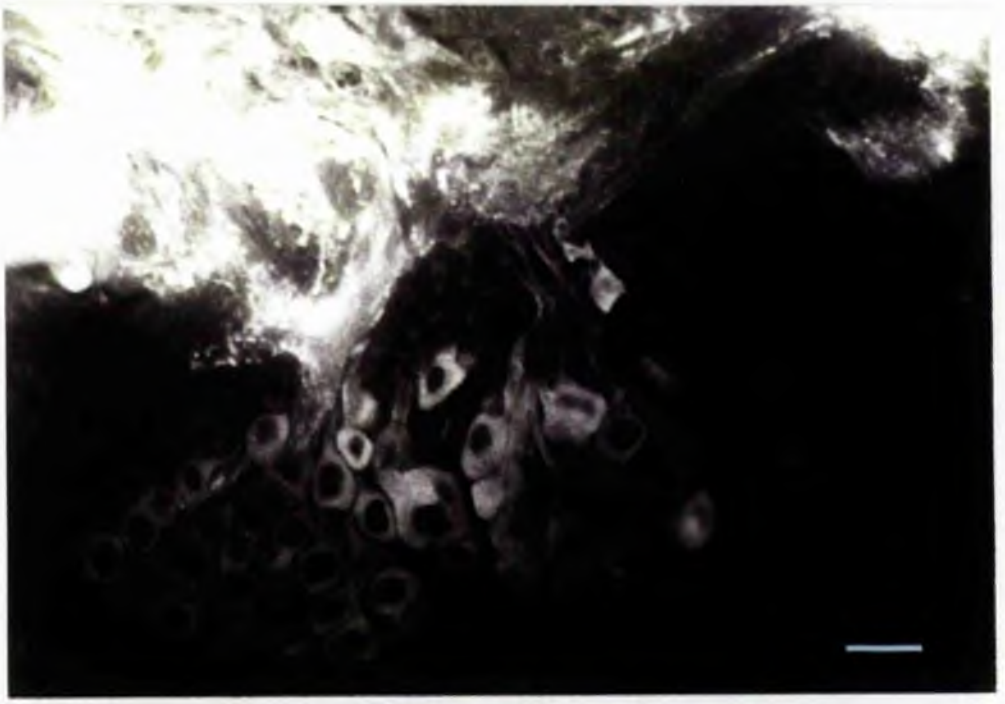

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In certain areas the fibres were closely packed together, giving a yellow colour. Collections of fluorescent fibres and varicosities often gave the appearance of complex monoamine pathways passing through or across different regions of the various ganglia.

Of interest was the appearance of green fluorescent fibres in the walls of the two large statocysts situated on each side of the pleural-pedal connectives (fig 32). This fluorescence was only associated with the outside wall of the statocyst.

3. Buccal ganglia

These are a pair of smooth oval ganglia connected to one another and to the cerebral ganglia by connectives. Nerve cells ranging up to 160μ in diameter are arranged peripherally around a central neuropile. The neuropile, as in other ganglia, showed many green fibres and varicosities (fig 33). Only very few fluorescent cells were present. These neurons were green in colour and were small, except for one neuron (60μ in diameter) which was located laterally on the ventral surface of each ganglion.

4. Heart

The heart consists of a thin walled fragile auricle and a large muscular ventricle. Monoamine

Fig 33. Buccal ganglia showing amine-fluorescence in the neuropile regions (n). Most of the cells are non-fluorescent. The ventrally situated gut (g) contains much auto-fluorescent material (a).
(The bar represents 100μ).

Fig 34. Section through the heart of Limax maximus. Fluorescent fibres are more prominent in the auricle (A) compared with the ventricle (V) regions.
(The bar represents 100μ).

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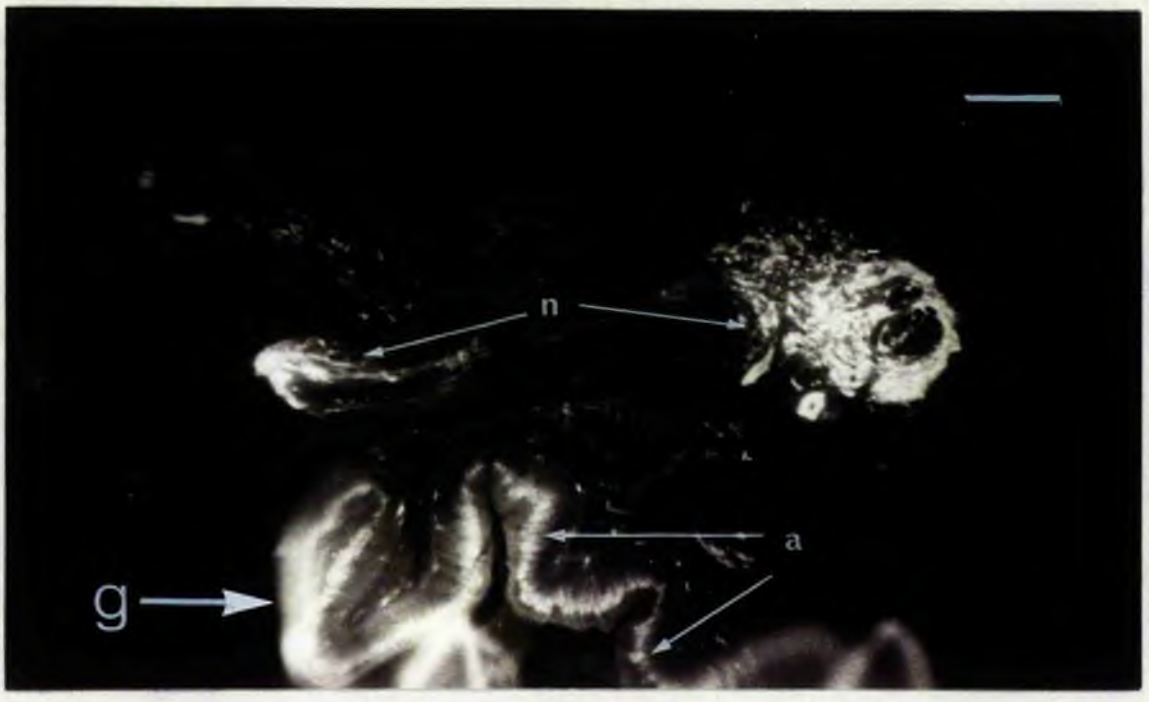

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fluorescence was confined only to nerve fibres which often appeared beaded. The fluorescent fibres were present in all areas of the heart, but the auricle possessed a greater number of monoamine-containing fibres than the ventricle (fig 34). No monoamine-containing cell bodies were observed in the heart. Unlike the fluorescence in the central nervous system which was generally either green or yellow in colour, the fluorescence associated with the heart was yellow-green in appearance. This probably represents a mixture of primary catecholamines and 5-HT, as in the snail heart (see page 100).

3. Integument

Green fluorescent processes (parts of neurons) were scattered throughout the integument and underlying musculature. Large numbers of these processes were especially prominent in the tentacles, facial grooves and buccal regions. At least two types of fluorescent processes could be discerned; fine structures, which generally occured singly and passed from the base to the surface of the integument (fig 35) and thicker, less regular structures which were often located in large numbers near the base of the integument (fig 36). It was not possible to trace any connections between the different fluorescent structures. The muscular layers

Fig 35. Section through the integument in the buccal region. Nerve branches are either irregular in shape (n) and scattered, or more uniform in structure (f) often occurring singly and passing from the base to the surface of the integument.
(The bar represents 100μ).

Fig 36. Another section through the integument. A number of nerve bundles (n) are located at the base of the integument.
(The bar represents 100μ).

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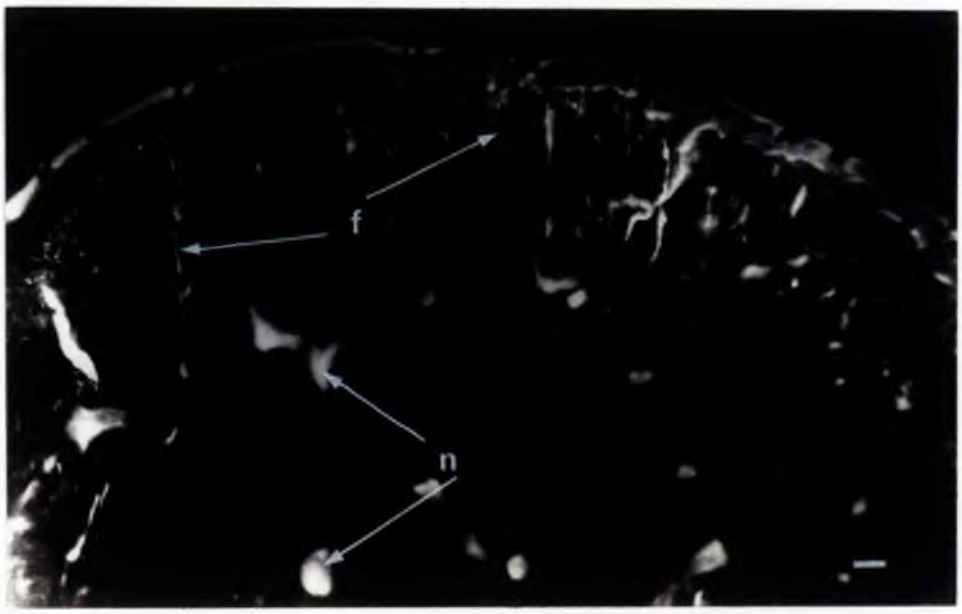

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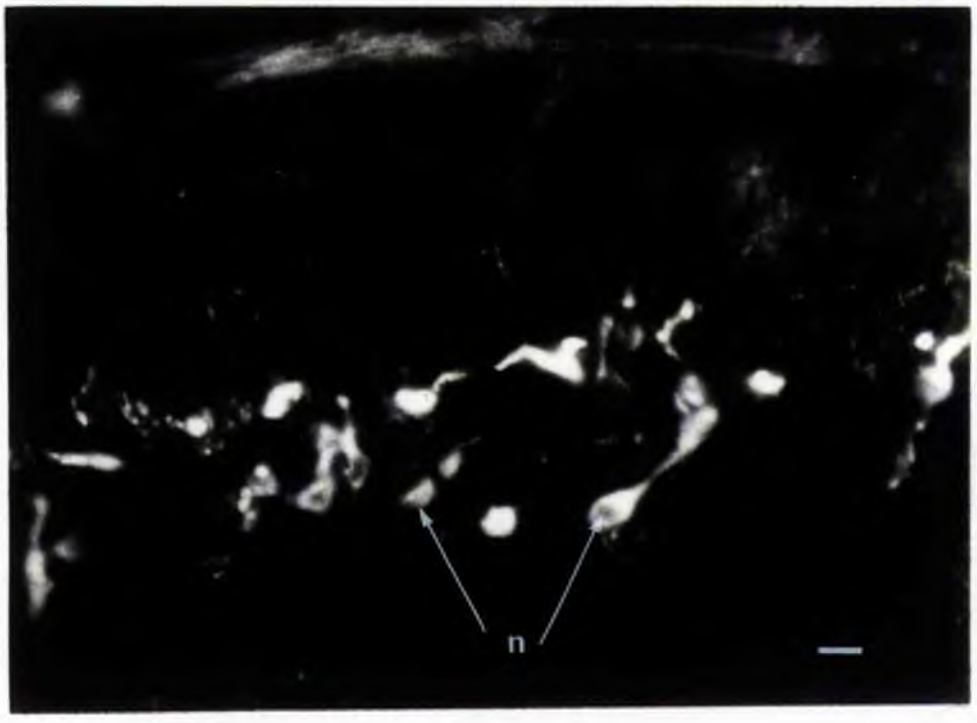

beneath the integument contained very few fluorescent processes compared with the integument.

6. Tentacles

The slug has two pairs of tentacles. The upper or optic tentacles are retractible and reversible, with bundles of retractor muscles. There are two nerves from the central nervous system which run along the length of each of the optic tentacles (see fig 37). No monoamine fluorescence was associated with the optic nerve, although some fine green processes were associated with the base of the retina of the eye (fig 38), to which the optic nerve connects. In contrast, the tentacular nerve and the digitate ganglion into which the nerve emerges (see fig 39) contained many green fluorescent fibres and varicosities. It is probable that the fluorescent varicosities localised in the neuropile region of the digitate ganglion connect with the processes observed in the integument (fig 40). The retractor muscles of the tentacles, especially in their basal regions were innervated with green amine-containing fibres. Occasionally intense red or dark green autofluorescence was observed in certain regions of the optic tentacles.

The lower pair of tentacles is also retractible, but has no eyes or digitate ganglia. The retractor muscles

Fig 37. Diagrammatic representation of an optic tentacle of a stylommatophoran pulmonate (from Lane 1964).

Fig 58. The eye of Limax maximus showing amine processes associated with the base of the retina (r).
(The bar represents 100μ).

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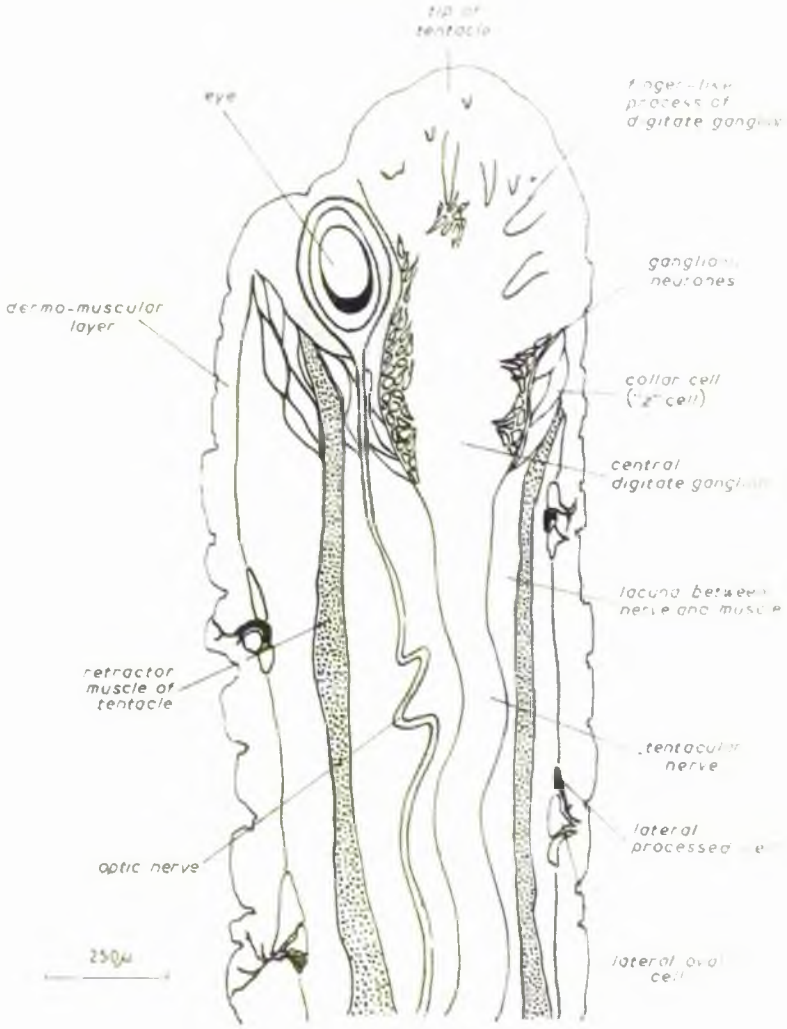
tip of tentacle
eye
dermo-musculor layer
collar cell
central digitate
retractor muscle of tentacle
optic nerve
250µ

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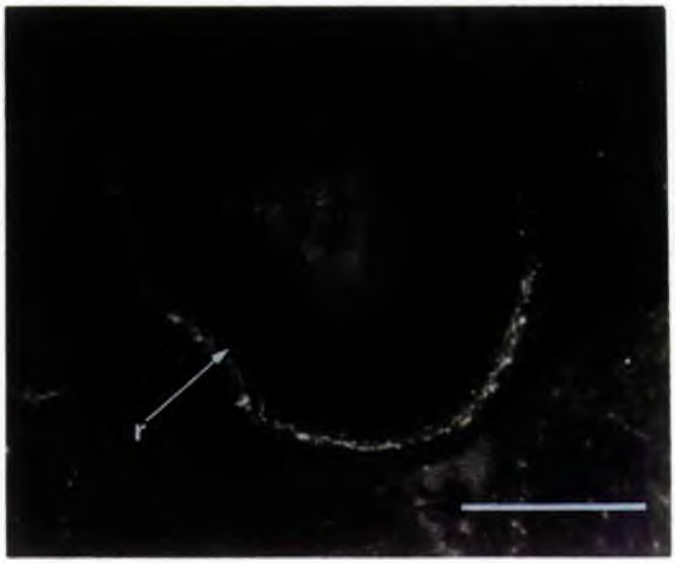

Fig 39. The optic tentacle of the slug. Amine nerve fibres are present in the digitate ganglion (d) and also prominent at the base of the integument (i). Fluorescence is also associated with the tentacular nerve (tn). (Each bar represents 100μ).

Fig 40. The tip of an optic tentacle showing an amine process (p) in the integument connecting with the digitate ganglion (d). (The bar represents 100μ).

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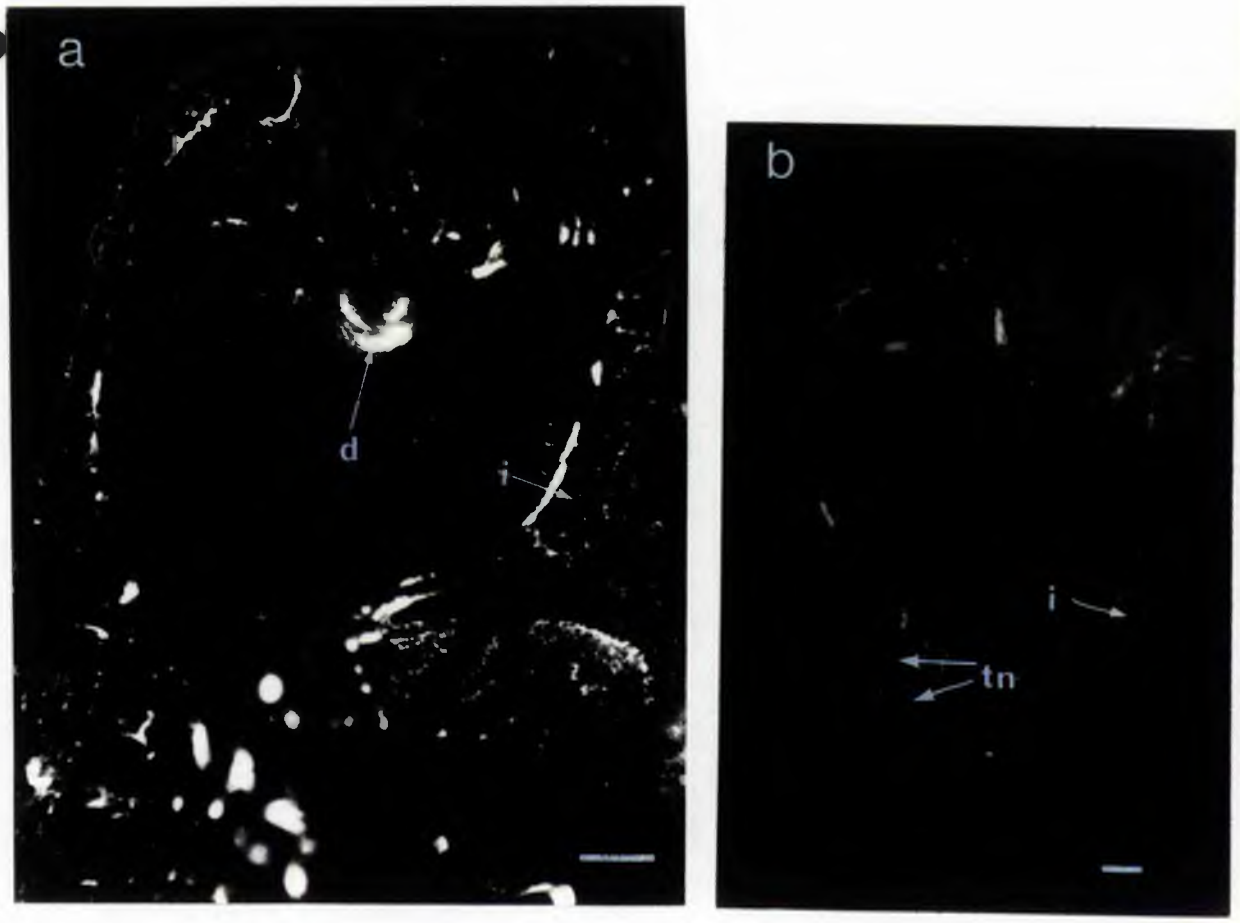

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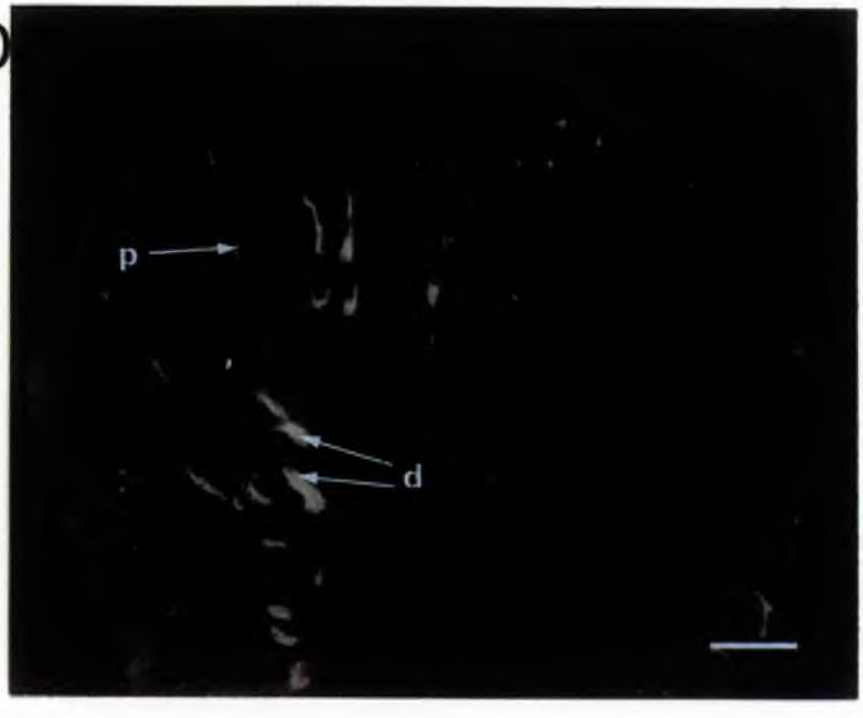

and integument of the tentacles contained many green fluorescent fibres and processes (fig 41). The tentacles are probably concerned with chemoreception, for their walls display folds which greatly increase the surface area.

7. Cephalic, Pharyngeal and Tentacular muscles

The cephalic retractor muscle arises from beneath the extremity of the mantle and divides into the pharyngeal and two tentacular branches. A sparse distribution of monoamine-containing fibres was located in each of the muscles (fig 42). Generally the nerve fibres followed the direction of the muscle fibres. In serial sections delicate varicose axons could often be traced for long distances close to the muscle fibre in a way suggesting muscular innervation. Monoamine varicosities were often concentrated in certain parts of the musculature, which indicates that there may be special areas of innervation. Fluorescent nerve cells were absent from all areas of muscle studied. Green autofluorescence was associated with the part of the tentacular muscle which joins the labial lobes. Otherwise the muscles were devoid of naturally fluorescent structures.

8. Penis retractor muscle

The penis retractor muscle is a stout band of muscle arising from the left side of the foot of the

Fig 41. Part of the lower tentacle of *Limax maximus*. Many amine-containing fibres (arrows) are scattered throughout the integument. Note the wall of the tentacle displays folds which greatly increase the surface area. (The bar represents 50μ).

Fig 42. Section of pharyngeal retractor muscle showing a large amine-containing fibre (arrow). (The bar represents 50μ).

Fig 43. Section of penis retractor muscle which contains many fluorescent fibres (arrows). (The bar represents 100μ).

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vestigial small columnella retractor, and is attached terminally to the penis sheath. Yellow-green monoamine-containing fibres were scattered throughout the muscle, (fig 43) but certain parts of the musculature were richly supplied with such fibres and presumed terminals. There was very little natural fluorescence associated with the musculature, and fluorescent nerve cell bodies were absent.

9 The Foot

The sole of the slug's foot has a uniformly pale appearance in life and secretes a colourless iridescent mucus. In the fluorescent microscope a small number of pedal gland ducts could be clearly seen because of their naturally fluorescent green secretion. These ducts were richly innervated by green amine-containing fibres (fig 44).

10. Kidney

This is a large brown organ lying close to the heart. Sections of the kidney tissue showed a number of tubules. The lumen of each tubule contains a large yellow autofluorescent granule. Tissues treated with formaldehyde vapour showed yellow-green fluorescence in the wall of the lumen. After sodium borohydride treatment the autofluorescent granules disappeared as did the specific fluorescence caused by exposing tissue to formaldehyde gas. However, only the

fluorescence associated with the wall of the lumen was restored when sections were again exposed to formaldehyde gas. From these results it is concluded that monoamines are located in the wall of the kidney tubules, but their role in this situation is not clear.

11. Alimentary Canal

The walls of the alimentary canal are richly innervated (see Bullock and Horridge 1965), and some of them exhibited amine-fluorescence. However it was found that a detailed study of monoamine distribution was not possible because of the abundance of autefluorescence materials which appeared yellow-green in the fluorescence microscope.

12. Reproductive organs.

The distribution of monoamine-containing neurons in the reproductive organs is complex, and it would require a separate study for a detailed description. Yellow and green neurons in the form of delicate fibres, some of which were varicose, were located in the ovotestis, albumen gland, sperm duct and oviduct (see fig 45). Naturally fluorescent materials were observed abundant.

DISCUSSION

The distribution of monoamine-containing neurons in the slug Limax maximus will be discussed from two points of view: (1) the relationship between the amine neurons and known data concerning the distribution of small granular

Fig 44. Section of the slug's foot showing amine fluorescent fibres (arrows) in the integument. Notice pedal gland (p) is richly innervated by green fluorescent fibres(arrows). (The bar represents 50μ).

Fig 45. The reproductive apparatus, showing monoamine fluorescence (arrows). (a) A section through the junction of the receptaculum seminis and vagina (b) A section through the vagina (c) A section through the common genital opening. Notice several auto-fluorescent (au) structures in the system. (The bar represents 100μ).

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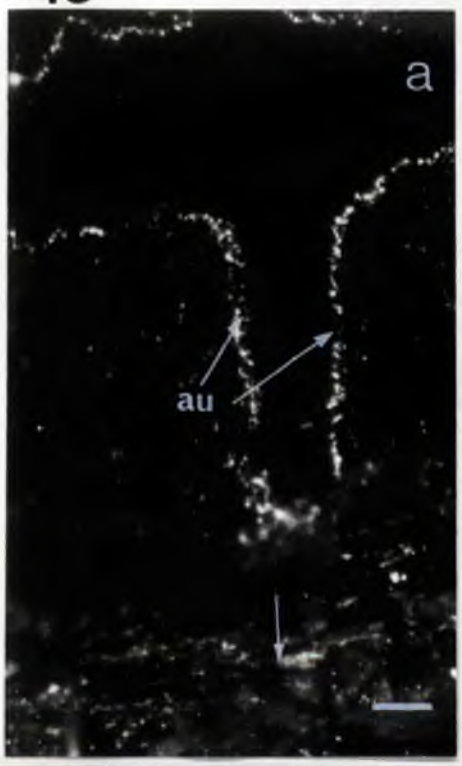
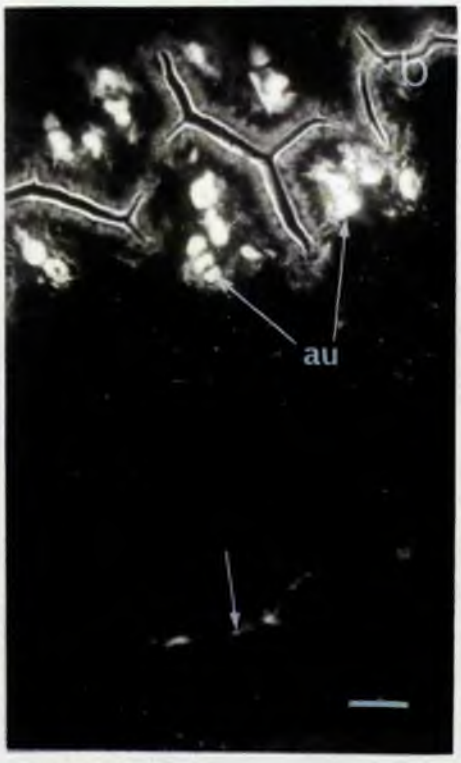




vesicles, which are thought to represent the sub-cellular sites for the monoamines; (2) the distribution and functional significance of the monoamines.

1. Monoamine distribution and granular vesicles

All the evidence available indicates that small granular vesicles, when present in nervous tissues of gastropod, represent sites for the localisation of different monoamines (see page 33). Only a certain number of cell bodies (Schmekal and Wechsler 1968) and axons in the neuropile regions (Gerschenfeld 1963) of gastropod ganglia contain small amine-type granular vesicles. Similar granular vesicles also occur in gastropod heart tissues (Amoroso Baxter, Chiquoine, and Nisbet 1964; Cottrell and Osborne 1969a and 1969b), penis retractor muscle (Schöite 1963 and Foh and Bogusch 1969), tentacular muscles (Rodgers 1968), the foot musculature (Rodgers 1969), the statocyst (Coggeshall 1969) and the retina of the eye (Eakin and Brandenburger 1967). It is clear that the distribution of small granular vesicles observed by the above workers in this and other gastropod tissues closely corresponds to the localisation of monoamine-containing neurons in the slug, Limax maximus

2. Distribution and functional significance of monoamines

The distribution of 5-HT and primary catecholamine

neurons in the central nervous system of Limax maximus in similar to that described for Helix aspersa (Sedden, Walker and Karkut 1968), but dissimilar was the fact that a collection of 5-HT neurons was not observable in the cerebral-pedal connectives. It is interesting to note that the giant 5-HT cell and associated cluster of catecholamine-containing cells which are located in each meta-cerebral ganglion of Limax maximus are also present in Limax flavus, Arion ater, Agriolimax reticulatus, Limnea stagnalis, Viviparus japonicus, Helix aspersa, Helix pomatia and Archatina fulica which I also studied. Thus there are similarities in the organization of monoamine-containing neurons in the central nervous systems of different species of gastropods studied. Another noteworthy feature about the distribution of monoamines containing neurons in Limax maximus is the relatively large number of fluorescent cell bodies observed in the meta- and meso-cerebral, visceral, pedal and right parietal ganglia. Nevertheless fluorescent fibres were seen in the neuropile in each ganglion, although in varying amounts. Serial sections of the ganglia gave the impression that monoamine fibres in the neuropile are arranged in a definite way. Often bundles of axons were interconnected to give a complex pattern. The lack of fluorescent cells in the buccal ganglia

and their total absence in the prote-cerebral ganglia show that these ganglia are primarily involved in non-monoaminergic mechanisms. The prote-cerebral ganglia are further distinguished by the absence of cell bodies greater than 15μ in diameter and by the direct synaptic contacts of many of the perikarya with axons. These last two characteristics were also observed by Zs-Nagy and Sakharov (1969) in other species of gastropod.

The yellow-green fluorescent varicosities and fibres in the heart of the slug are similar to those present in the heart of Helix pomatia (see page 98). It seems likely that 5-HT is also the cardio-excitatory transmitter in the slug.

Catecholamine fibres and other presumed neuron processes in the integument and in some of the underlying musculature are similar to the amine fluorescence described as occurring in the cephalic tentacles of two species of prosobranch molluscs (Storch and Welsch 1969), and in the foot musculature of Helix pomatia (Rodgers 1969). Because the catecholamine fluorescence is especially abundant in the tentacles and integument in the anterior parts of the slug, it seems probable that the catecholamines are involved in sensory functions. Similar green processes have been described in the epithelium of sea anemones

(Dahl, Falck, Limdqvist and Mecklenburg, 1963b) and the earthworm (Rude 1966 and Myhrberg 1967). In Limax maximus, it would appear that fibres from at least some of the peripherally placed monoamine-neurons contribute to the intense green fluorescence observed in the neuropile regions of the different ganglia.

Green fluorescence indicative of primary catecholamines was also associated with the eyes and statocysts. The catecholamines in these locations may well be involved in the transmission of sensory information to the brain. It is of interest to note that the eye originates embryologically from an infolding of part of the integument. The function of primary catecholamines in the eye could also be to act as a local hormone which sets the eye into rhythm (Jacklett 1969) or to take part in a sensory mechanism (Naka and Brandenburger 1967). There is some evidence that catecholamines are involved in the preception of geotaxic information in molluscs (Sweeney 1968), which would account for the amines' localisation in the statocysts.

There is proof that certain muscle preparations respond to monoamines. It has been shown that 5-HT relaxes molluscan muscle, e.g. the anterior byssus retractor muscle of Mytilus (Twarog 1954), penis retractor muscle (Jaeger 1963),

radular retractor muscle (Fange and Mattisson 1958) and pharyngeal retractor muscle (Kerkut and Cottrell 1963). The occurrence of yellow-green fluorescence in the gut musculature, retractor muscles and body wall musculature of the slug suggests that the monoamines are important in the nervous processes involved in the control of at least some of these muscles.

It appears that monoamines are involved in the release of substances from glands. Large numbers of varicosities are associated with the pedal glands of the foot, which secretes a mucus. This could also explain the presence of monoamine fibres in the walls of the lumen of the kidney tubules and those located in the reproductive apparatus. Mann 1963, suggested that 5-HT plays a part in the ejaculation of seminal fluid from Octopus vulgaris. Furthermore, an adrenergic innervation is important for the emission of semen from the human epididymis through the ductus deferens (Baumgarten, Falck, Holstein, Owman and Owman 1968).

There exists a limited number of functional possibilities for the monoamines. The localisation of monoamine-containing neurons in the central nervous system implies that monoamines are involved in specific neural functions. DA, NA and 5-HT are almost certain to have independent roles. From the data and arguments presented,

it appears as if primary catecholamines play a role in sensory and perhaps some associated functions, and also probably in secretory activities such as the release of mucus. The indolalkylamine 5-HT is often associated with nerves innervating musculature. This monoamine is known to have a potent effect upon isolated muscle preparations. It is suggested that 5-HT is primarily involved in either regulating or relaxing musculature. Myhrberg (1966) had thought that yellow fluorescence is associated with motor functions.

SUBCELLULAR LOCALISATION OF 5-HT IN AN IDENTIFIABLE GIANT CELL IN LIMAX MAXIMUS

INTRODUCTION

It is important to know the subcellular localisation of 5-HT in nervous tissue in order to interpret its physiological role. Previous work on gastropod tissue (see page 33) shows that 5-HT is probably localised in small granular vesicles, but confirmation of this has proved difficult, mainly because it has not been possible to study tissue known to contain 5-HT and no other monoamines.

In this respect, the giant pair of yellow fluorescent perikarya located in the meta-cerebral ganglia of Limax maximus (see fig 29) appeared ideal objects for study. Each cell measured up to 180µ in length and could easily be seen, in situ, with a x30 magnification binocular microscope.

It was decided first of all to obtain further evidence for the presence of 5-HT in the neurons before proceeding to study the amine's localisation. This was done by observing the effects of different drugs on the amine fluorescence of the giant cells and further by assaying isolated neurons on the Helix pomatia heart, a preparation known to be very sensitive to 5-HT.

MONOAMINE HISTOCHEMISTRY OF GIANT NEURONS

Although the formaldehyde-induced fluorescence in the giant neurons was characteristic of 5-HT, it was necessary to ensure that no other amine was also present, especially since Kerkut, Sedden and Walker (1967) have provided evidence which indicates that some neurons in the brain of Helix aspersa contain both DA and 5-HT. This was done by examining the nature of amine fluorescence in these cells from a number of slugs pretreated with different drugs which are known to interfere with monoamine levels. The results of such experiments are summarised in fig 60.

The intensity of the yellow fluorescence of the giant neuron was reduced by pretreatment with p-chlorophenylalanine, which blocks the conversion of 5-HTP to 5-HT (see page 44) though it was not altered by α-methyl-m-tyrosine, an inhibitor of tyrosine hydroxylase (Spector, Sjoerdsma and Undenfriend 1965). Injections of the 5-HT precursor 5-HTP increased the intensity of fluorescence, but there was no change in the appearance of the fluorescence after injecting the catecholamine precursor, DOPA.

These experiments therefore provided extra evidence that 5-HT is present in the giant cells and furthermore that the neurons do not contain any primary catecholamines.

BIOLOGICAL ASSAY OF GIANT CELL, 5-HT

1. Preparation of Extracts

Individual giant cells were dissected under a microscope in the following way. First connective tissue layers from and around the area of the central nervous system containing the cell were carefully removed. Then the neuron was gently sucked up into a minute reservoir of a finely drawn out glass pipette, and subsequently transferred to a small test tube containing Meng's saline or distilled water.

Extracts of neurons were prepared by homogenising 6-10 cells in either Meng's saline or distilled water. Control extracts were prepared from the same number of similar sized neurons of the buccal ganglia which contained no monoamines. These solutions were assayed for their 5-HT content using the isolated heart of the snail Helix aspersa.

2. Isolation of Snail Heart

The calcareous shell of the Helix aspersa was removed and the heart exposed by carefully cutting first along the mantle wall and then the pericardium. A thin split was then made at the junction of auricle and vena cava into which a narrow cannula was inserted. A

thread was carefully passed underneath the auricle and cannula and used to tie the cannula into the auricle. The venae cavae leading into the auricle were then severed, the cannula and heart lifted gently, and the heart finally freed from the animal by cutting away a little patch of kidney into which ran the aorta.

The cannula, with the heart joined to it, was anchored in a clamp, as shown in fig 46. A bent entomological pin and a piece of thin cotton were used to attach the heart lever to the small piece of kidney attached to the aorta (see fig 46). The position of attachment of the heart to the lever was adjusted so as to give a suitable amplification of the heart-beat on a smoked kymograph drum. The perfusion pressure for the heart beat was kept constant by regulating the flow of the fluid so that the overflow arm was full, but not running over.

3. The Effect of Monoamines on Snail heart.

All the amines tested upon the isolated heart had the effect of increasing the amplitude and frequency of the heart beat. A comparative effect of monoamines in exciting an isolated Helix heart is shown in fig 47. The primary catecholamines are about 10,000 -

Fig 46. Diagram of the isolated snail heart preparation.

A - Inflow from perfusion system.

B - Overflow arm.

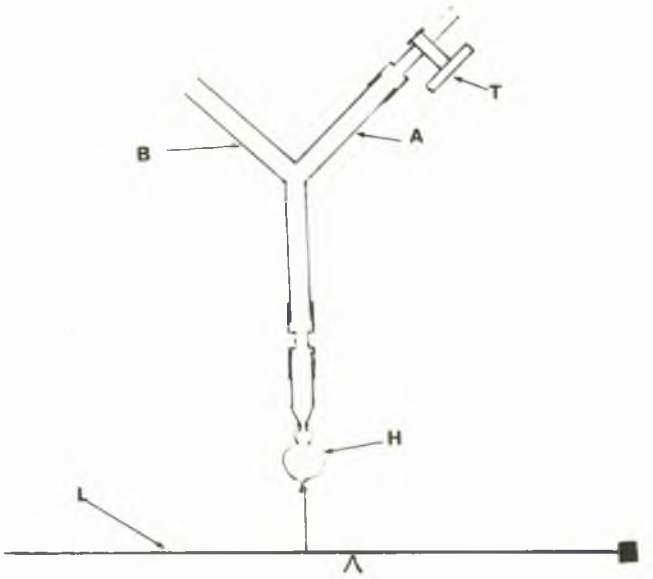
L - Heart lever.

H - Isolated heart.

T - Tap to adjust rate of perfusion of saline.

Fig 47. Response of an isolated Helix pomatia to different concentrations of monoamines.

46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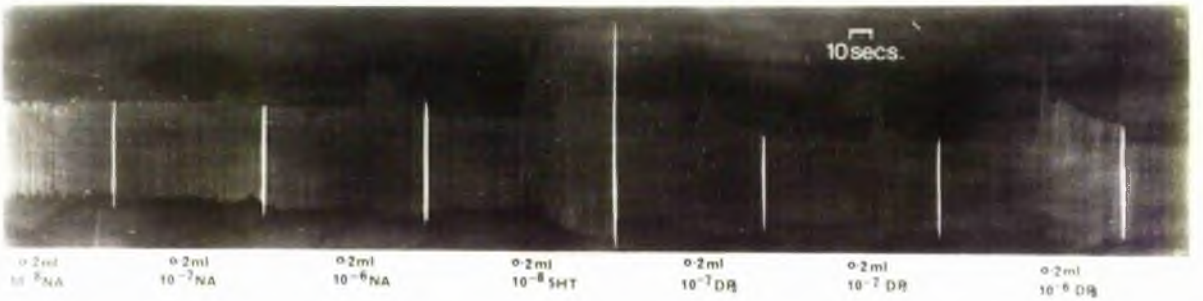

10 secs.
0·2ml
10⁻⁷NA
0·2ml
10⁻⁶NA
0·2ml
10⁻⁸5HT
0·2ml
10⁻⁷DB
0·2ml
10⁻⁷DB
0·2ml
10⁻⁶DB

100,000 times less potent than 5-HT in stimulating the heart.

The effect of monoamine precursors, as compared to the amines in exciting the isolated heart of Helix pomatia are summarised in Table 5.

4. Effect of Cell Extracts

Addition of giant cell extracts to an isolated snail heart led to an increase of amplitude and frequency of the heartbeat. The effect was very much like that caused byalow concentration of 5-HT. As shown from fig 48, these responses were antagonised by perfusing the heart with 1 litre 10^{-6} M bromo-lysergic acid diethyl-amide (BOL). Extracts from control cells had no effect.

In each of seven assay experiments, extracts consisted of 6-10 cells in 1 ml solution. From the results the 5-HT content was estimated to vary from 6-8 ng/cell.

FINE STRUCTURE OF GIANT CELL

1. Method

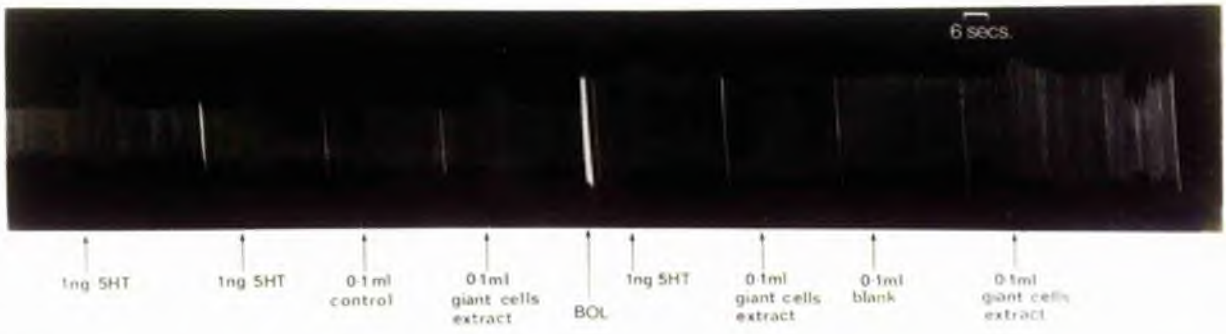
From each cerebral ganglion a small part containing a single giant 5-HT neuron was rapidly dissected and fixed in one of the different solutions (a) 1% OsO_4 buffered by 0.3M cacodylate, pH 7.2, for 1 hour. (b) 1% OsO_4 buffered by 0.2M veronyl acetate, pH 7.4, for 1 hour (c) 2% glutaraldehyde buffered by

TABLE 5. Summary of minimum concentration of amine (in grams) required to excite the perfused Helix heart.

Fig 48. Responses of an isolated Helix pomatia heart to 5-HT, an extract prepared from individually isolated giant 5-HT cells, and to a similarly prepared extract of an equivalent number of non-fluorescent cells (control). All the excitatory responses are blocked by the drug BOL (bromo-lysergic acid diethylamide).

TABLE 5

Substance	Minimum amount of drug, dissolved in Meng's saline and then injected into the cannula of a perfused Helix pomatia heart
DOPA	10^{-4}
5-HTP	10^{-5}
AD	10^{-7}
NA	10^{-7}
DA	10^{-8}
5-HT	10^{-12}



0.3M cacodylate, pH 7.2, for 2 hours. (d) 2% glutaraldehyde with 0.3M cacodylate buffer, pH 7.2, for 1 hour then followed by 1% OsO_4 and 0.4M cacodylate for 1 hour.

Fixed tissues were de-hydrated in acetone solutions and embedded in Araldite. To locate the giant neuron, serial sections (0.5μ thick) were cut on the LKB ultramicrotome stained with toluidine blue and observed with the light microscope. Thin sections of the neuron, gold-silver in colour, were cut, mounted on uncoated grids, double stained with lead citrate (Reynolds 1963) and 2% uranyl acetate solution and examined with the electron microscope.

2. Results

The most conspicuous organelles in the cytoplasm are small vesicles and lysosome-like structures (fig 49). Most of the small vesicles have electron dense core centres. The average diameter of these granular vesicles is 60-120 nm, and the appearance of their electron dense core varies with the nature of fixatives used (fig 50). The centres of these vesicles are particularly opaque in tissues fixed with either glutaraldehyde and OsO_4, or glutaraldehyde alone. These granular vesicles are most abundant in areas of the cell containing granular-endoplasmic reticulum and in the axon hillock. Often the

Fig 49. Part of the perikaryon of the giant 5-HT cell in the meta-cerebral ganglion of Limax maximus. In (a) a number of lysosome-like particles or cytosomes (l), vesicles (v), Golgi fields (g), mitochondria (m) and the nucleus (n) are marked. Fig (b) is a higher magnification of another part of the perikaryon which shows clearly the presence of granular vesicles (v) in the cytoplasm. The endoplasmic reticulum (e) is also marked. Tissue was fixed in glutaraldehyde and osmium and stained with lead citrate and uranyl acetate. (Each bar line represents 1 μ.

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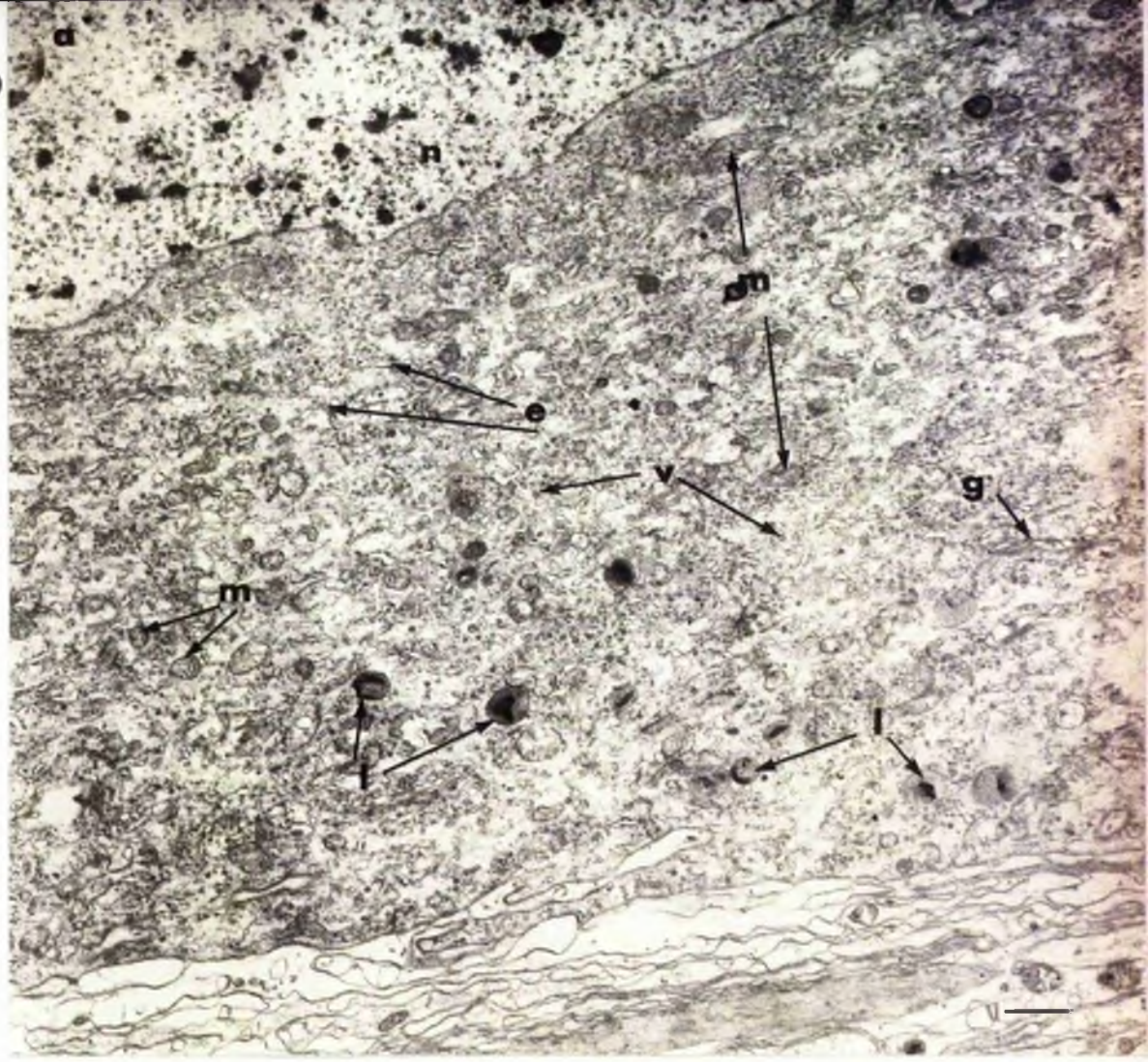


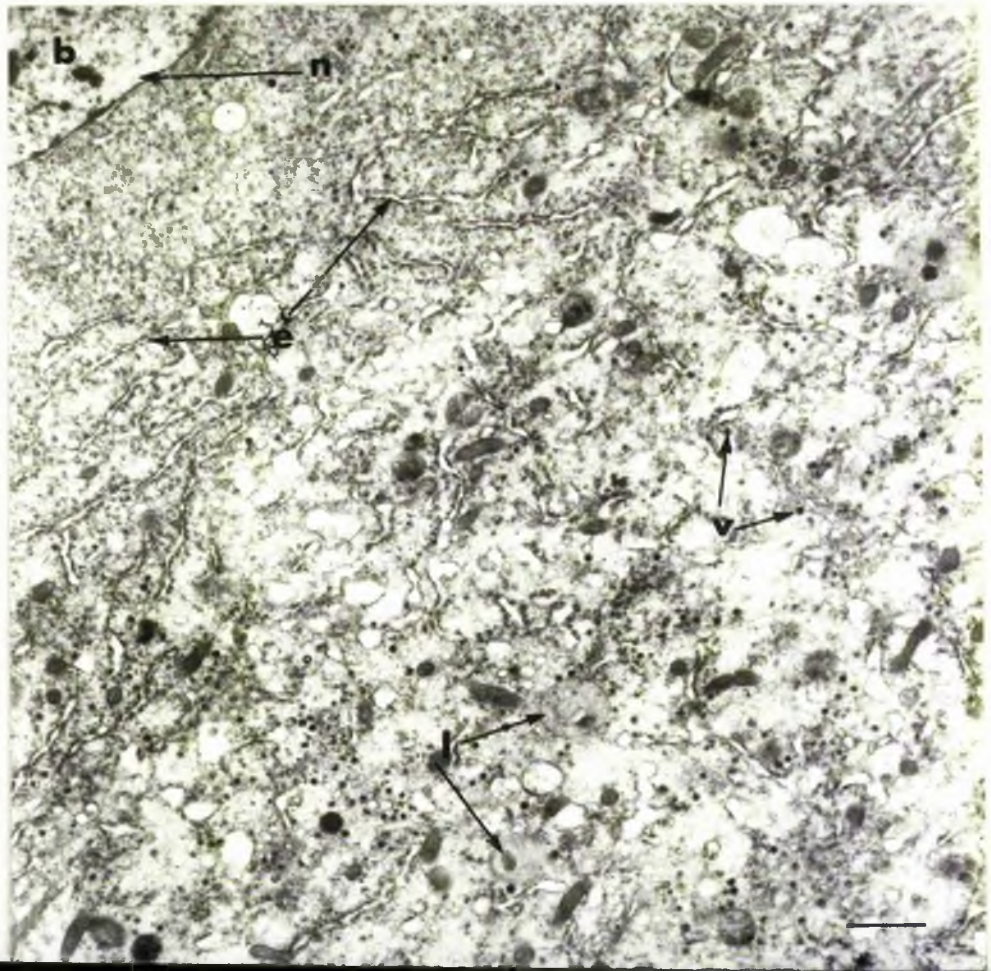


Fig 50. (a) A low magnification, and (b) a higher power view of granular vesicles (arrows) in the cytoplasm of the giant neuron. Notice the electron dense granules are in the core of the vesicles. Tissue was fixed in glutaraldehyde and osmium and stained with lead citrate and uranyl acetate.
(The bar line in (a) represents 0.8μ and that in (b) 0.2μ).


a


b

vesicles are particularly associated with the Golgi fields (fig 51) and to a lesser extent with the lysosome-like structures (fig 52).

The appearance of lysosome-like particles varies (fig 53). Typically these organelles are surrounded by smooth membranes, and vary from 0.3 to 1.5μ in diameter. Their centres normally seem to be homogeneous but sometimes have laminated membranes or vesicles. Often irregular electron dense material is associated with the organelles.

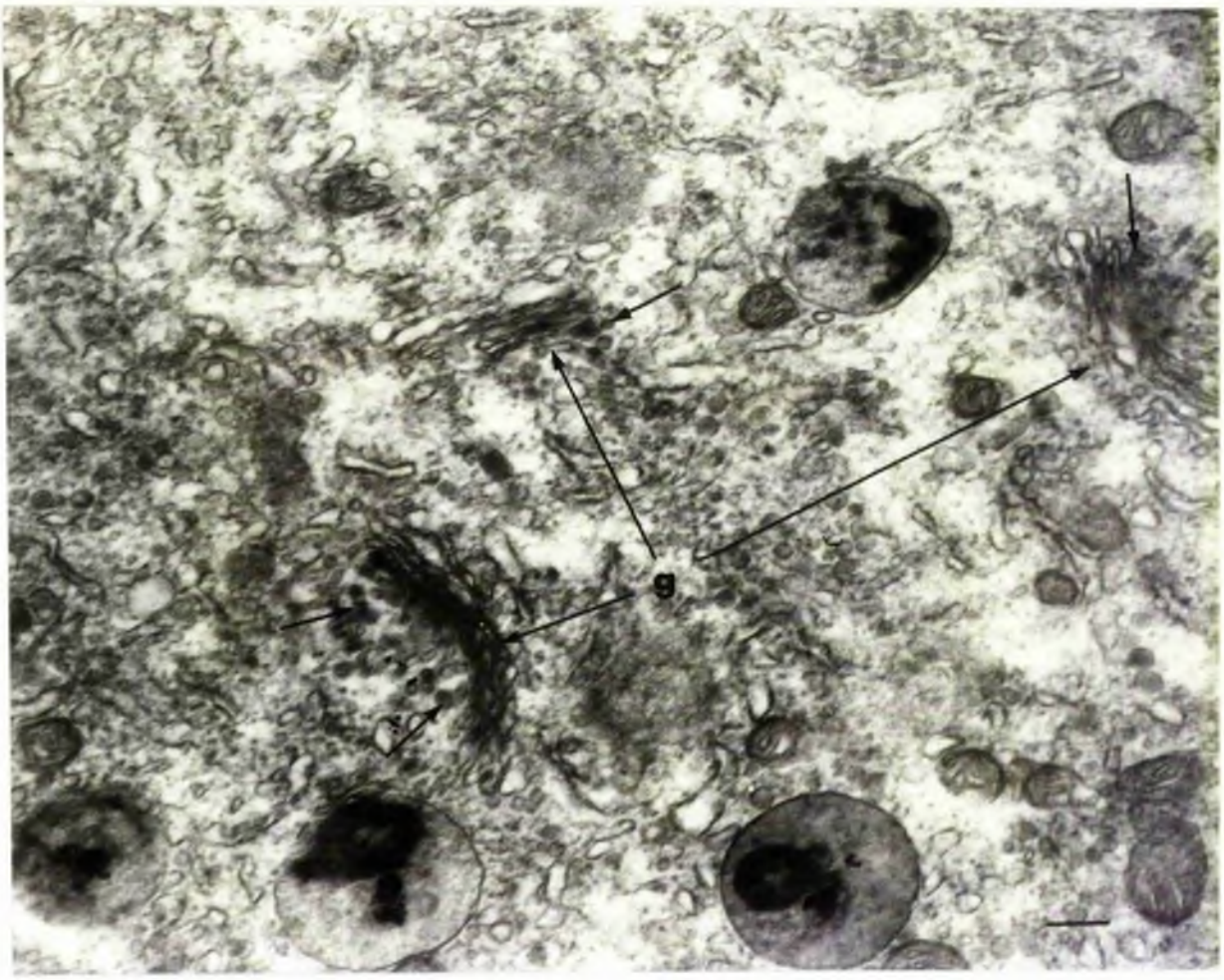
A number of other structures are present in all areas of the cytoplasm, but arranged in no apparent order. Mitochondria are narrow and tubular in appearance and have simple cristae (figs 52 & 53). Golgi fields (figs 49 & 51), often associated with granular vesicles, are scattered in all areas of the cytoplasm. Other conspicuous structures are ribosomes, crystalloid bodies, and a few "multi-vesicular" bodies. There are no pigment granules or granules normally associated with neurosecretory substances in the cytoplasm.

The nucleus is very large, forming approximately one fifth of the cell. It consists of a homogeneous spongy caryoplasm without a nucleolus, but containing some chromatin layers. The wall of the nucleus exhibits numerous, irregular deep folds (fig 54).

Fig 51. High power view of part of the perikaryon showing a number of Golgi fields (g) and their association with vesicles (arrows) Tissue was fixed in osmium and stained with lead citrate and uranyl acetate. (The bar line represents 0.2μ).

Fig 52. Micrograph showing a cytosome (c) containing laminated membranes and vesicles (v). Tissue was fixed in glutaraldehyde and osmium and stained with lead citrate and uranyl acetate. (The bar line represents 0.2μ).

51



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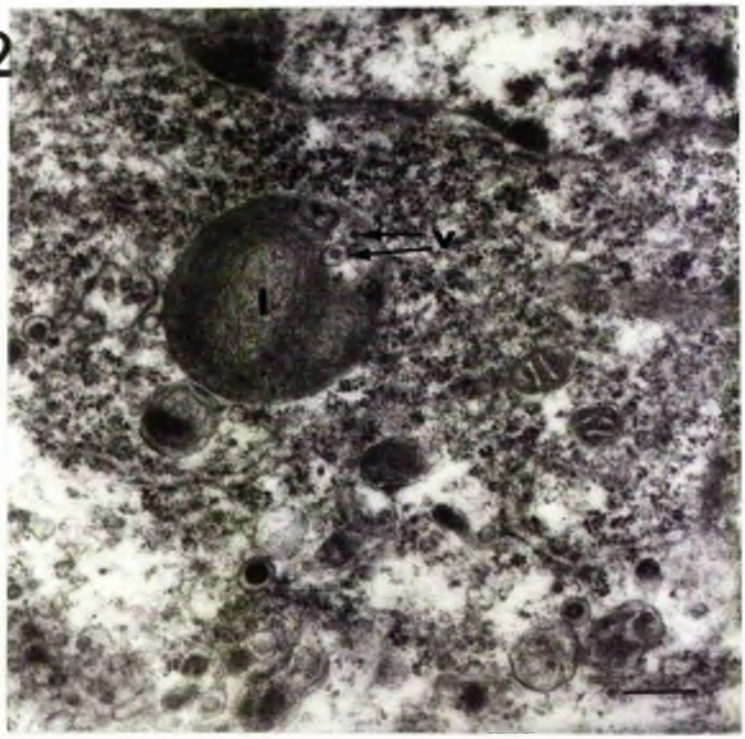
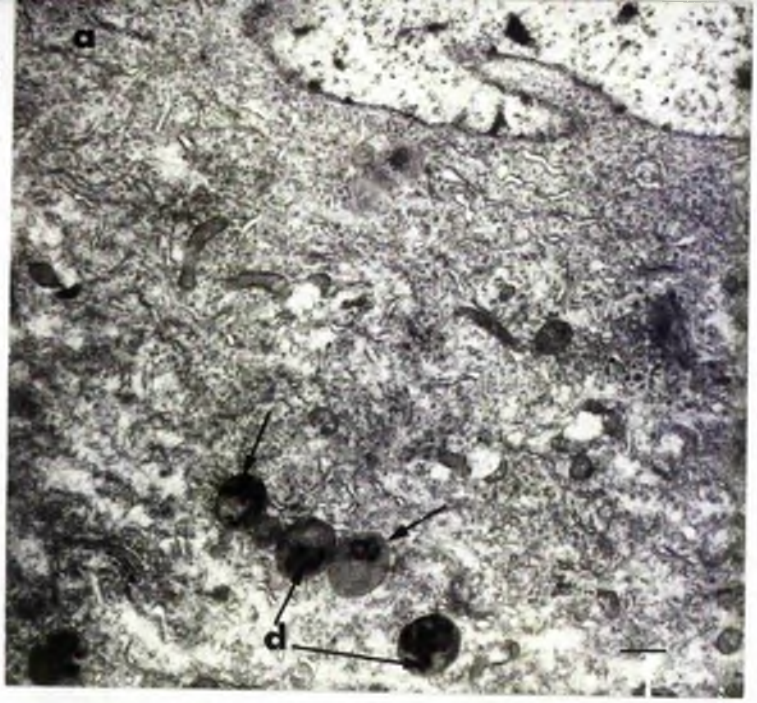


Fig 58. The different appearance of cytosomes (arrows) in the cytoplasm. In (a) the organelles contain much electron dense material (d). In (b) some of the cytosomes are clear and furthermore have laminated membranes (lm). Fig (c) shows some of the organelles to contain granular vesicles (v) as well as complex membranes (lm). All tissue was fixed in glutaraldehyde and osmium and stained with lead citrate and uranyl acetate. (The bar in (a) represents 0.5μ, that in (b) 0.2μ and that in (c) 0.2μ).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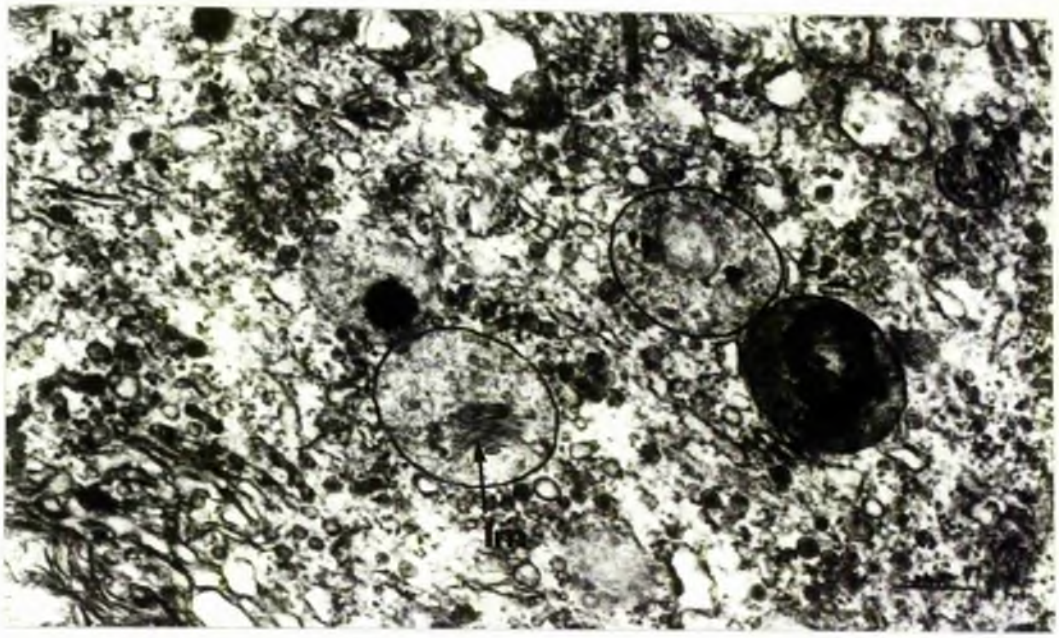
d


b
lm

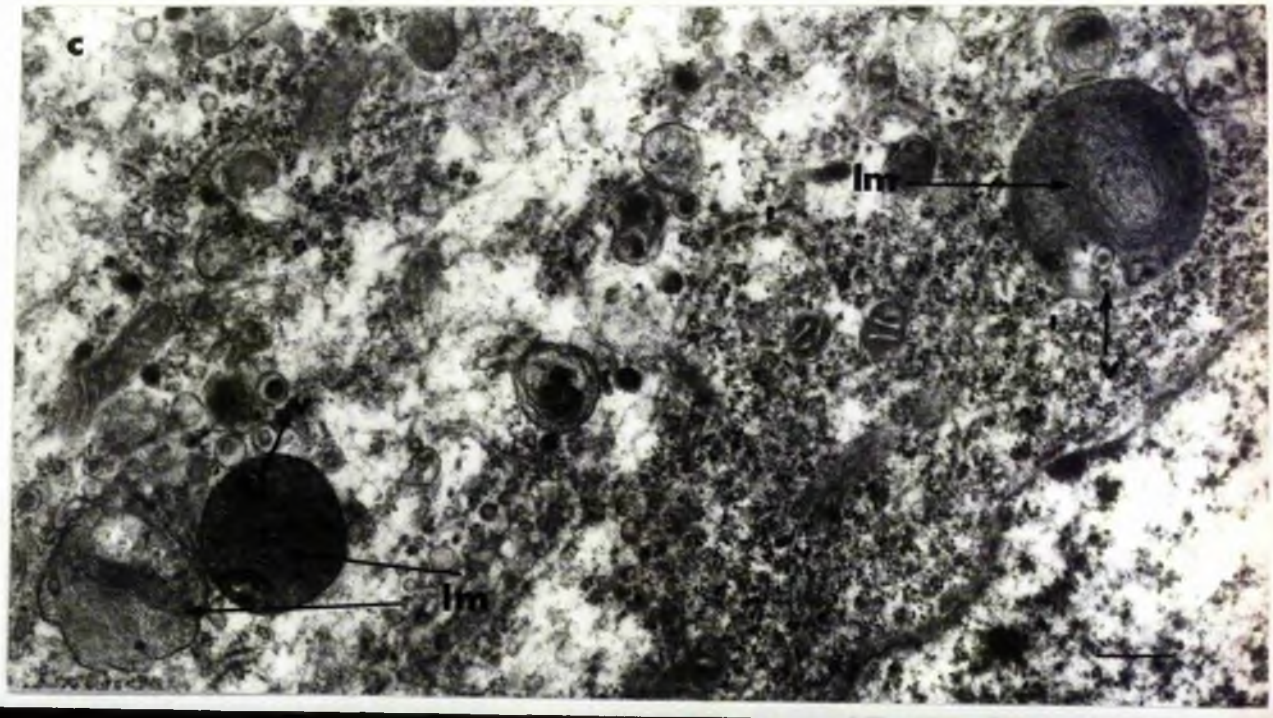
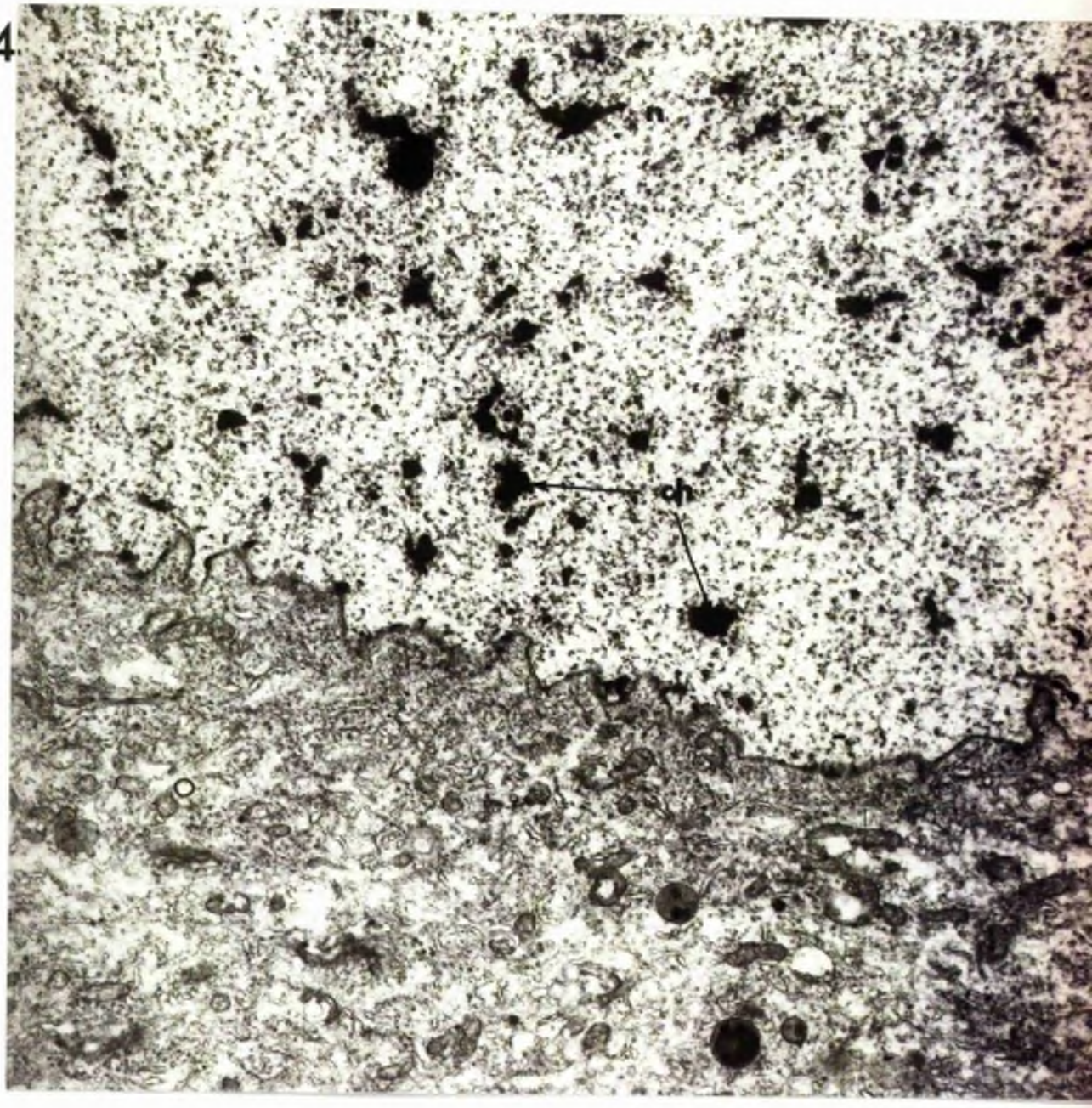

c
lm
lm

Fig 54. Part of the perikaryon showing the large nucleus (n) of the cell. The nuclear membrane displays many folds, and chromatin (ch) can be clearly seen in the nucleoplasm. Tissue was fixed in osmium and stained with lead citrate and uranyl acetate.
(The bar line represents 0.5μ).


n
ch

Electron Microscopical Localization of 5-HT

In recent years methods have been developed for the electronmicroscopic demonstration of primary catecholamines in cells containing large amounts of amines such as in the adrenal medulla (e.g. Wood and Barnett, 1964; Tramezzani, Chiocchio and Wassermann 1964; and Coupland and Hopwood 1966). The reactions for the demonstration of primary catecholamines are based on the fact that an insoluble complex is formed between aldehyde and amines and that, upon exposure to metal-containing oxidising agents such as dichromate (Wood and Barnett 1964), ammoniacal silver (Tramezzani, Chiocchio and Wassermann 1964) and osmium tetroxide (Coupland and Hopwood 1966), a dense precipitate identifiable under the electron microscope is formed. Subsequent studies have shown that 5-HT can also be demonstrated by these methods. Using glutaraldehyde fixation followed by either treatment with potassium dichromate (Wood 1965, 1966) or ammoniacal silver carbonate (Cannata, et al 1968), 5-HT was demonstrated as a dense precipitate under the electron microscope.

The exact chemical reactions for the demonstration of monoamines, in situ, are not clear. Experiments with tissue models (see Coupland and Hopwood 1966; Arnold and Hager 1968 and Solcia, Sampietro and Capella 1969) show that

the mechanisms are pH dependent and very complex and that an immediate Schiff base formation between amine and aldehyde, may not be involved.

Present methods do not histochemically differentiate between the monoamines. However, primary catecholamines appear to reduce ammoniacal silver or ferricyanide solutions at a faster rate than 5-HT (Cannata, et al. 1968), a phenomenon which can be used to indicate the nature of monoamines.

METHODS

Small pieces of tissue of a cerebral ganglion containing a giant neuron were fixed in 10 ml 2% glutaraldehyde plus 2 ml 0.3M cacodylate buffer, pH 7.4 for 2-3 hours at 4°C. Tissue was then washed in 0.12M cacodylate buffer for 15 minutes at room temperature.

Some fixed tissue were placed for 20 hours in 1.5% potassium dichromate plus 0.5% sodium sulphate buffered to pH 4 with 0.05M acetate buffer (after Wood 1965, 1966). Other glutaraldehyde fixed tissues were processed according to the method described by Cannata, Chiocchio and Tremezzani 1968):

(a) placed in freshly prepared ammoniacal silver carbonate (prepared as described in Charlton and Drury 1957).

(b) washed in distilled water for 5 minutes.

(c) placed for 5 minutes in 1% sodium thiosulphate.

Tissues histochemically stained by both methods, were subsequently washed in distilled water, dehydrated in a graded acetone series and embedded in Araldite.

Thin gold-silver sections were cut, mounted on uncoated grids, and examined in the unstained condition or when stained with lead citrate and uranyl acetate.

RESULTS

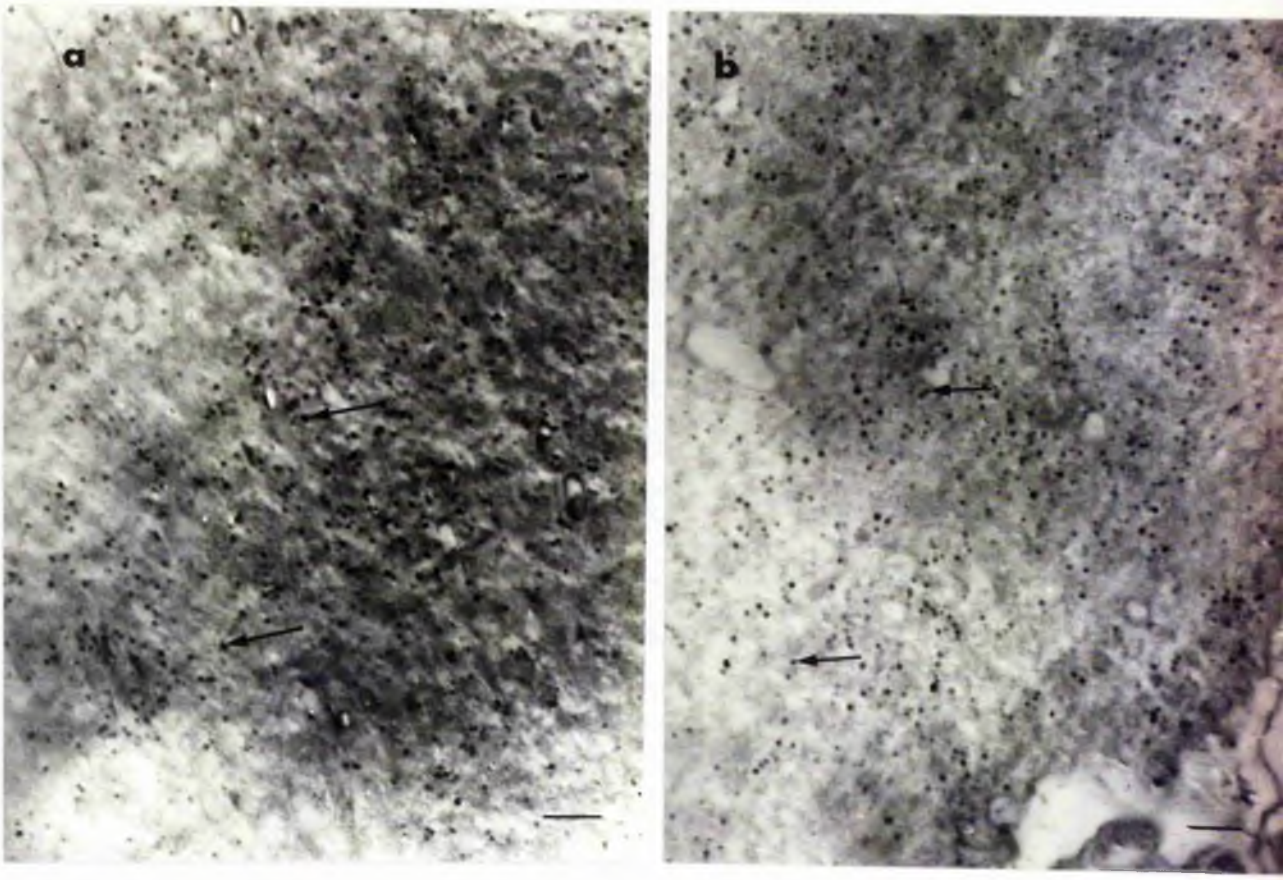
Ganglia removed from slugs during the summer months and processed by the Wood's method showed deposits of electron dense material of the same size as the cores of the granular vesicles (fig 55). Although much of the cellular detail could not be observed in sections of tissue processed by this histochemical method; membranes could often be observed to encircle each granular deposit. (fig 56). Tissue from slugs which had been injected with reserpine drastically reduced the number of granules observed. It was therefore concluded that 5-HT is associated with these small granular vesicles.

Tissues examined earlier in the year (in springtime) provided evidence for an additional localisation of 5-HT. At this time tissues stained for the cytochemical localisation

Fig 55. Electron dense deposits (arrows) in cytoplasm of neuron representing sites for amine localisation. Tissue processed by Wood's method in summer months. (Each of the bar lines represent 0.1μ).

Fig 56. Part of cytoplasm of giant neuron processed by Wood's method and stained with lead citrate and uranyl acetate, in summer months. The electron dense deposits are shown to be localised in vesicles (arrows). (a) A low power view and (b) shows membranes surrounding the granular vesicles. (The bar line in (a) represents 0.8μ and that in (b) 0.2μ).

55



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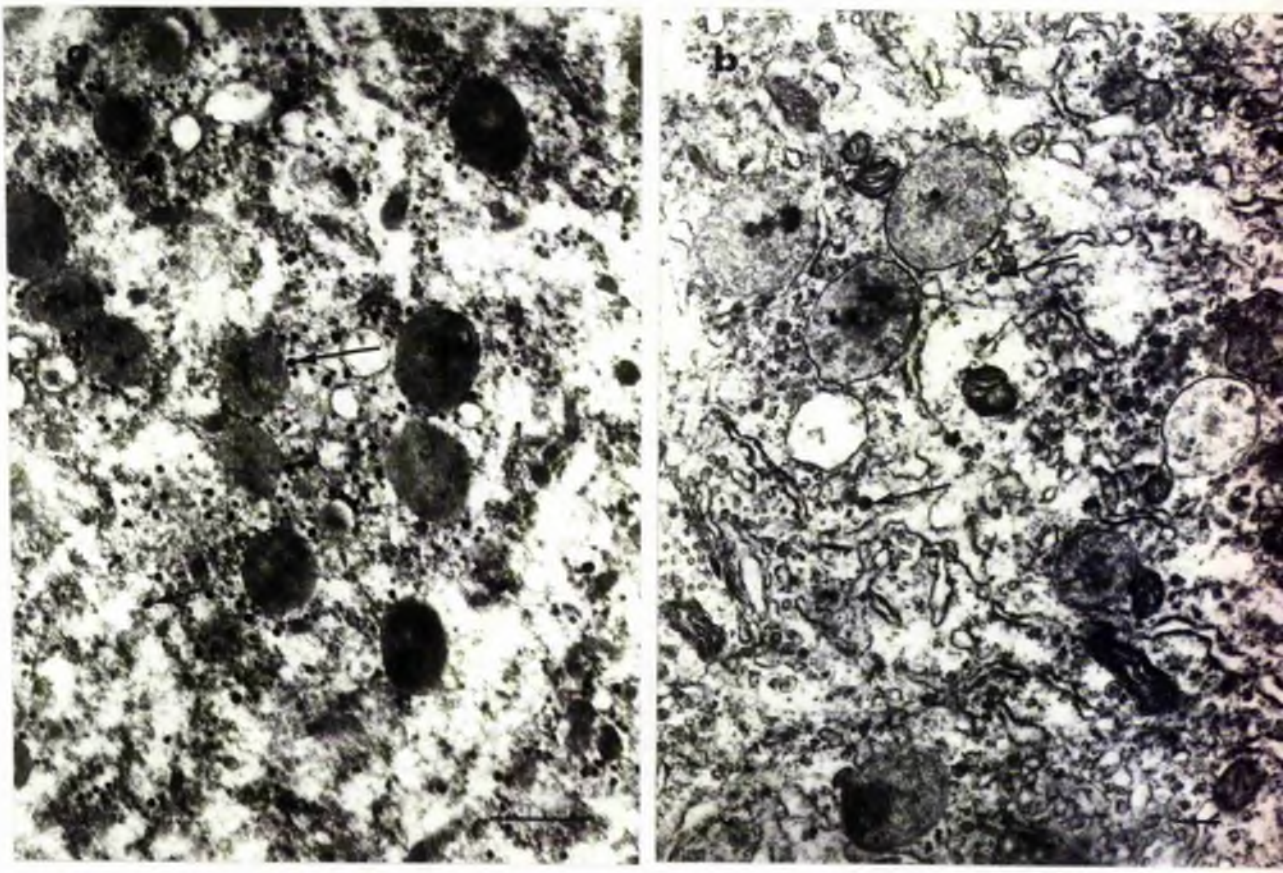


of amines showed dense reactive products in particles which resembled the lysosome-like structures (fig 57 & 58). Similar results were obtained with tissues processed by the methods of Wood and Cannata, although the reactive products appeared finer in tissue prepared by the later method. Tissues, processed for localisation of 5-HT by both of the methods, and then either slightly postosmicated or stained with uranyl acetate and lead citrate, provided confirmation for the localisation of reactive products in lysosome-like particles (fig 59).

It was estimated that only about one quarter of the total number of lysosome-like particles in the cell body reacted positively to 5-HT. Prior injection of reserpine drastically reduced the dense deposits in these organelles. Examination of tissues processed for cytochemical localisation of amines from slugs which had been injected with p-chlorophenylalanine (specifically depletes 5-HT, see page 44) provided confirmation for the localisation of 5-HT in granular vesicles and lysosome-like particles by the absence of dense deposits in the giant meta-cerebral neuron.

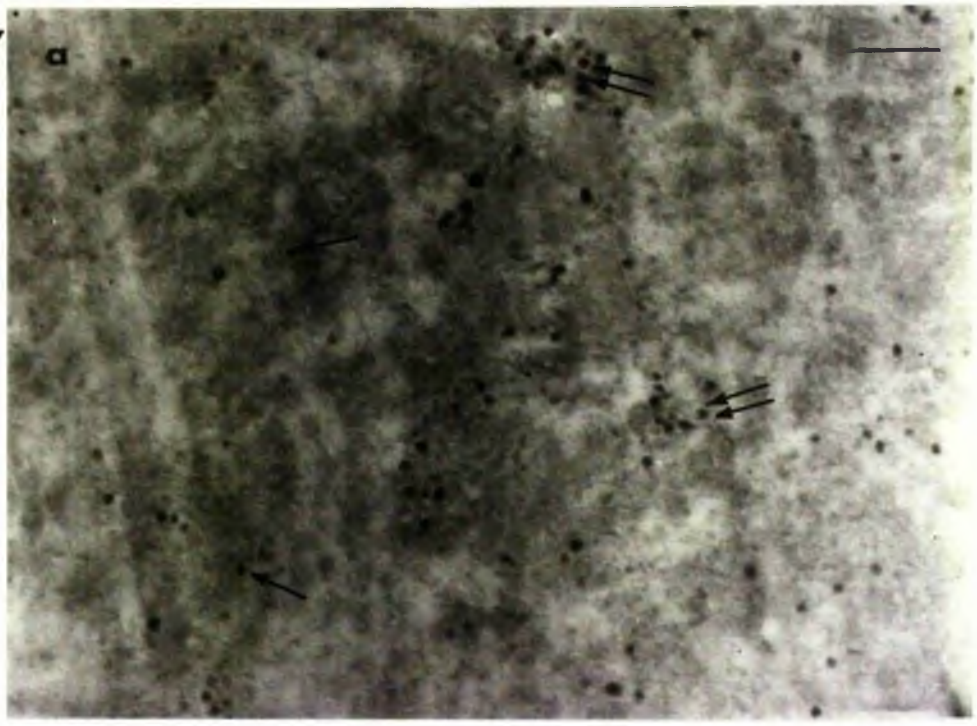
DISCUSSION

It is concluded for the following reasons that

Fig 57. (a) Electron dense deposits (arrows) in cytoplasm of neuron processed by the Wood's method in springtime. Notice aggregation of electron-dense deposits (double arrows). (The bar line represents 0.8μ).

(b) Tissue processed by Wood's method in springtime and stained with lead citrate and uranyl acetate. Electron dense deposits (arrows) are shown to be localised in vesicles and cytosomes. (The bar line represents 0.8μ).

57



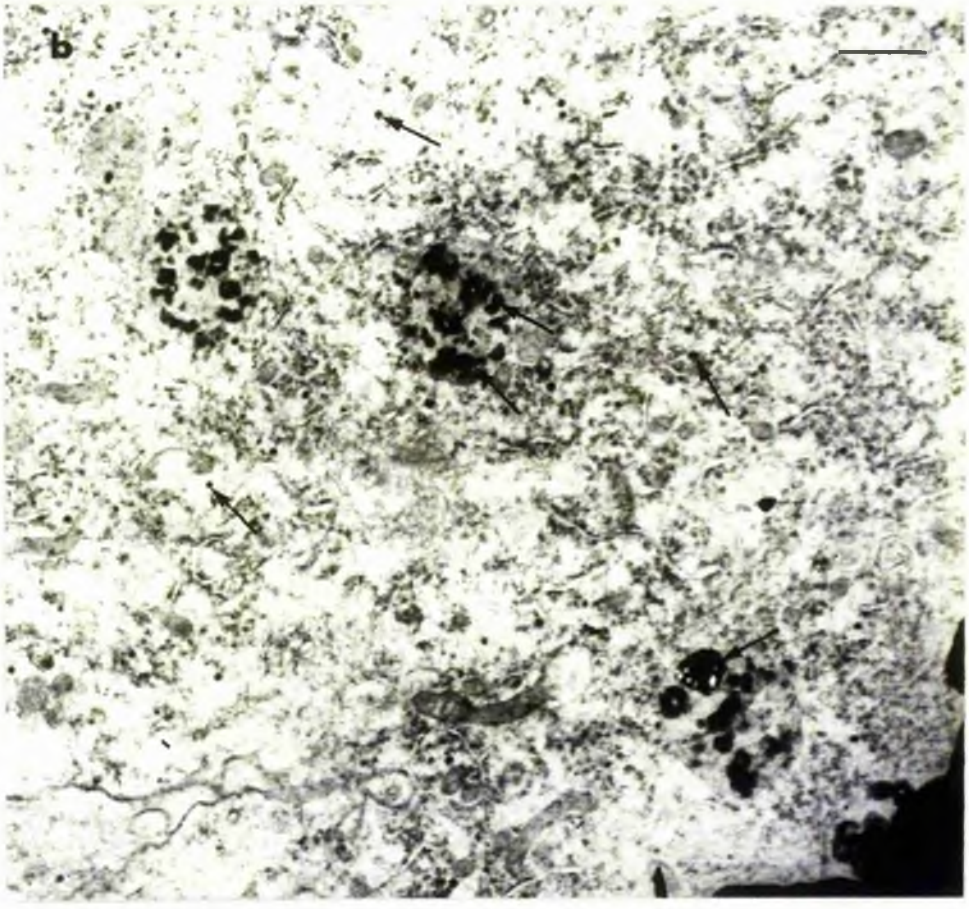
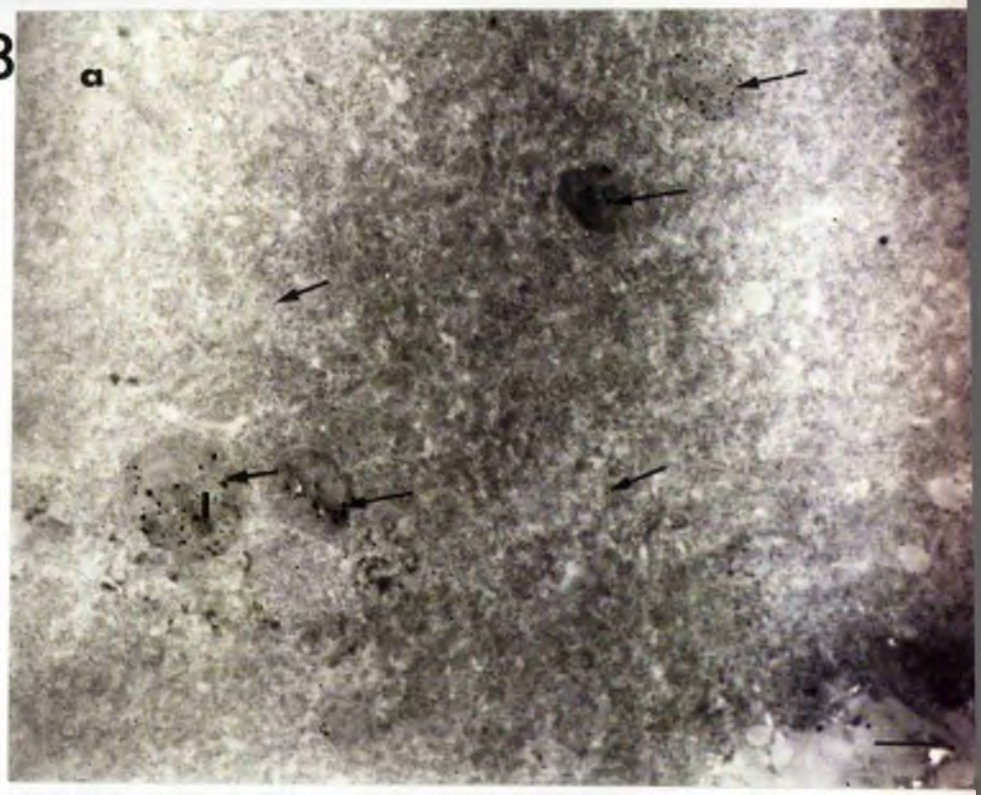


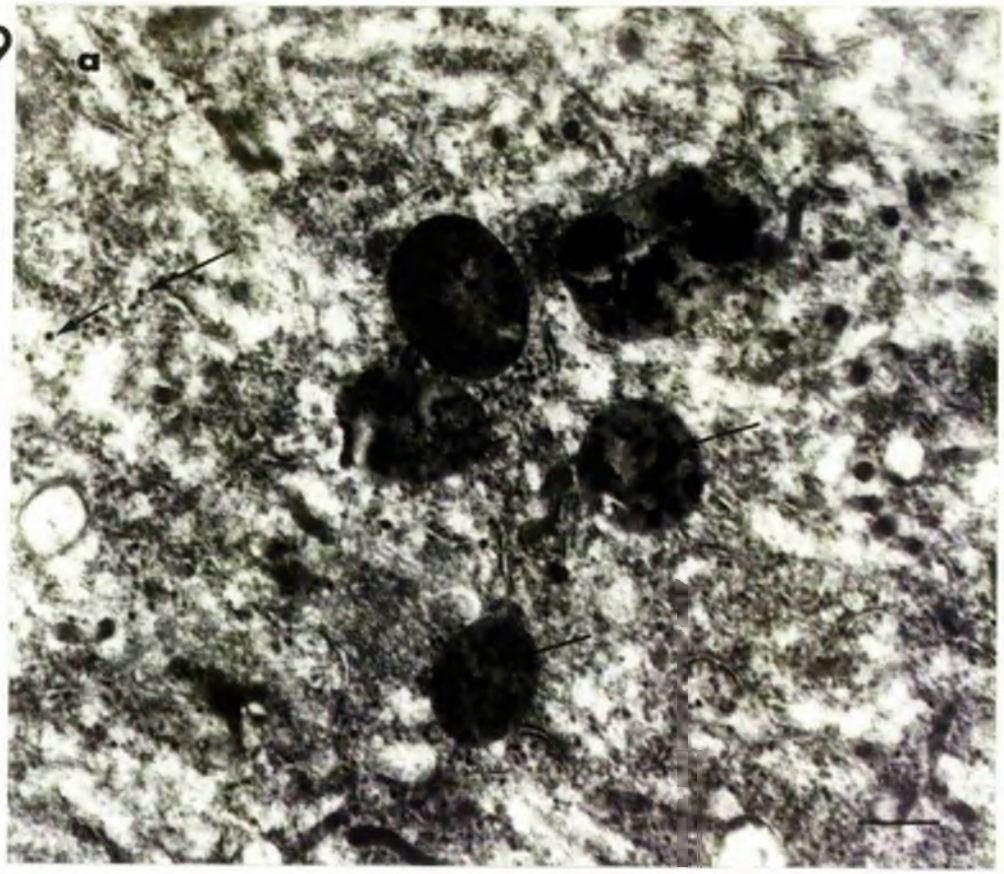
Fig 58. Electron dense deposits (arrows) in cytoplasm of neuron processed by the method of Carmata et al in springtime. (a) A low power view and (b) another section at a higher magnification. Staining indicative of amine particularly dense in particles which resemble cytosomes (1).
(Each of the bars represent 0.5μ).

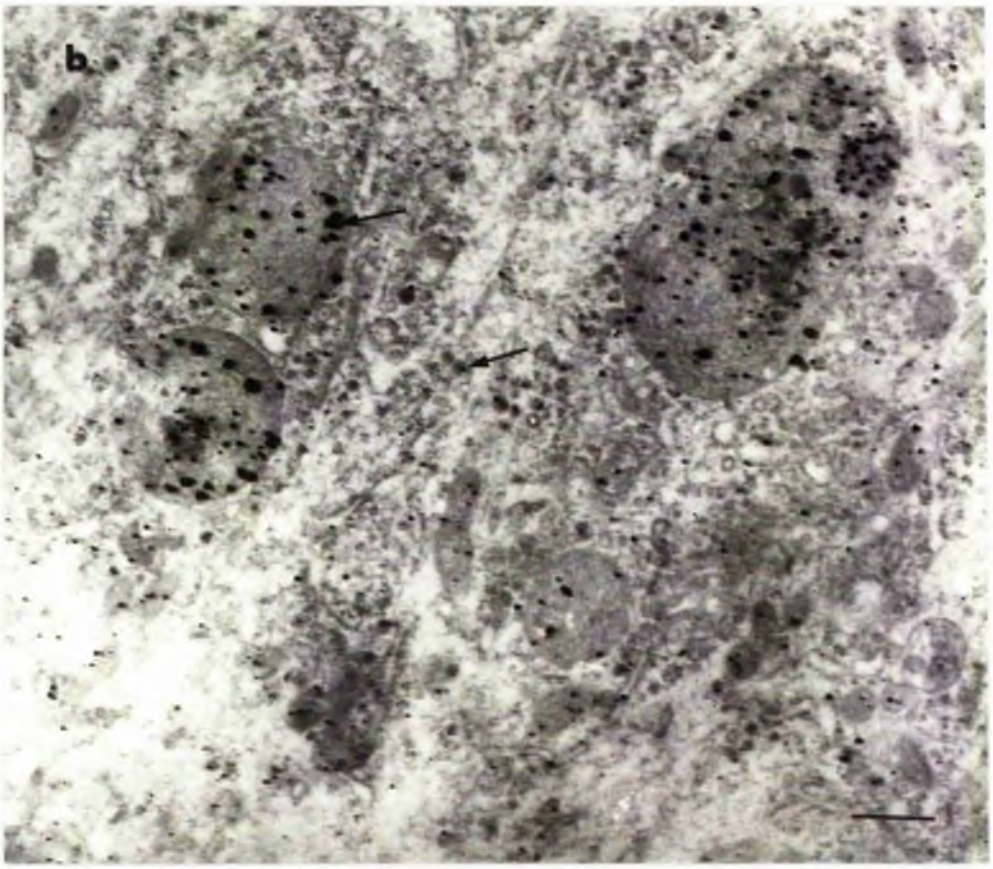

a


b

Fig 59. Tissue (in springtime) processed by (a) Wood's method and (b) Cannata's method, then slightly post osmicated and stained with lead citrate and uranyl acetate. Electron dense deposits (arrows), representing sites of amine localisation are in cytosomes and vesicles.
(Each of the bars represent 0.8 μ).

59





of all the monoamines, 5-HT alone was present in the giant cells: (1) The giant neurons fluoresced yellow after exposure to formaldehyde vapour, indicative of 5-HT, and the fluorophore formed was relatively unstable to ultra-violet irradiation. (2) Pretreatment of slugs with certain drugs known to interfere with the metabolism of different monoamines suggests that the yellow fluorescence in the cytoplasm of the neurons was derived solely from 5-HT (for summary see fig 60). (3) Extracts from giant cells excited the isolated Helix heart in the same way as pure 5-HT, and both of these responses could be blocked by perfusing the heart with BOL.

The fine structure of the 5-HT containing cells shows the cytoplasm to be characterised by a lack of highly organised endoplasmic reticulum. A number of organelles e.g. Golgi apparatus, mitochondria and multi-vesicular bodies, all possible candidates for 5-HT localisation were observed throughout the cytoplasm. The most conspicuous formations were however the lysosome-like particles and granular vesicles. The latter were especially clear in tissue fixed with glutaraldehyde and post-fixed with osmium tetroxide. The lysosome-like

Fig 60. Summary of the effects of various drugs on the yellow fluorescence in the giant neuron.

Name of Drug	EFFECTS	EFFECT ON YELLOW FLUORESCENCE OF GIANT CELL.
Reserpine	Depletes amines from molluscan nervous tissue (Mirroli & Welsh 1964)	All fluorescence eliminated.
p-Chlorophenyl-alanine	Reduces 5-HT content by inhibiting the enzyme tryptophan hydroxylase in vertebrates. (Koe & Weissman 1966)	Colour of fluorescent still yellow although intensity reduced
∝-methyl-m-tyrosine	Reduces CA content by inhibiting the enzyme tyrosine hydroxylase in vertebrates (Moore 1966)	No change in colour and intensity of fluorescence
5-HTP	Precursor of 5-HT in molluscs. (Welsh & Moorhead 1959)	Intensity of yellow fluorescence increased
DOPA	Precursor of CA's in molluscs. (Gardot 1963)	No change in colour and intensity of fluorescence
Nialamide	Monoamine oxidase inhibitor in vertebrates.	Slight increase in intensity of yellow fluorescence
MSD 1024	DOPA decarboxylase inhibitor in molluscs. (Kerkut, Selden & Walker 1967)	Yellow fluorescence very slightly reduced

particles in gastropod nervous tissue, although larger than "true" lysosomes, have a similar ultrastructure. These organelles have other similarities with "true" lysosomes, in that they have been shown to contain a paraldehyde fuchsin positive material (Stutinsky, Forte, Tranzer and Termimn 1963; Baranyi 1966) and to contain the enzyme acid phosphatase (Hook and Lane 1964). However, the particles differ from "true" lysosomes* in that they contain the enzyme succinic dehydrogenase (Nachlas, Tsou, Sousa, Chong and Seligman 1957; Burstone 1959) normally associated with mitochondria, and acid phosphatase only at certain times of the year (Baranyi 1966). It is for these reasons, (i.e. lysosome-like particles in gastropod nervous tissue have characteristics common to both lysosomes and mitochondria) that Holte, Brencker and Kuhlmann 1965, termed these organelles "cytosomes". Their possible functions in

* These are particles that range in size from 0.2-0.8μ in diameter. They are distinguished from other types of particles in the cytoplasm mostly on the basis of their biochemical properties, namely their content of enzymes which are primarily hydrolytic in function. Lysosomes have been demonstrated <u>in situ</u> with the electron microscope and stained to demonstrate their acid phosphatase activity. Morphologically lysosomes are still not adequately characterised.

molluscs have been discussed by Nolte, Breucker and Kuhlmann 1965; and Zs-Nagy 1967c. Lysosome-like particles or cytosomes have also been shown to occur in Annelids (Rochlich, Aros and Vigh 1962; Coggeshall and Fawcett 1963), Insects (Scharrer 1963) and certain vertebrates (Lemos and Pick 1966).

The results of electron microscopic cytochemistry using the method of Wood (1965, 1966), which is known to be relatively specific for certain amines in gastropod molluscs (Cottrell 1967; Newman, Kerkut and Walker 1968; and Cottrell and Osborne 1969b), and the method of Cannata, Chiocchio and Tramezzani (1968), show 5-HT to be localised in the small granular vesicles and cytosomes. The occurrence of the amine in these organelles would account for the speckled appearance of the yellow fluorescence in the cytoplasm of the giant neurons when processed for amine-fluorescence histochemistry. The localisation of 5-HT in the form of electron dense deposits varied according to the method used. Employing the technique described by Cannata, et. al. (1968), electron dense deposits are distinctly shown to occur in the homogenous areas of cytosomes. According to Cannata et. al (1968) the size of the electron dense deposit is influenced by the length of ammoniacal silver carbonate

treatment; all 5-HT deposits should be observed after treatment with solution for 30 minutes. However, even after prolonged treatment with ammoniacal silver carbonate for up to 90 minutes, deposits were so fine in the granular vesicles that they were often missed and were only seen in unstained sections at very high power. This method, therefore, proved more successful for localisation of 5-HT within cytosomes, which presumably means that when the amine is present in these structures its concentration is high when compared with that in granular vesicles.

Glutaraldehyde fixed tissue, treated with dichromate according to the method of Wood (1965, 1966), showed reactive deposits of 5-HT clearly in both granular vesicles and cytosomes. The amine was located in the core of granular vesicles of size 60-120nm in diameter. It has been shown by Coupland and Hopwood (1966) that monoamines localised in the form of electron dense deposits can be detected in tissues fixed with glutaraldehyde and post fixed in OsO_4. For this reason it was not surprising to find that the cores of granular vesicles appeared particularly dense in tissue fixed in this manner. The light brown material observed in granular vesicles of

tissue fixed in glutaraldehyde or OsO_4 and then stained, is presumably a result of proteins within the vesicles. Bloom and Aghajanian (1967) suggested that the dense content of vesicles is partly due to the protein matrix.

The localisation of 5-HT in granular vesicles which vary from 60-120 nm in diameter is consistent with other work. All the literature on the subcellular localisation of monoamines in the molluscs infers that 5-HT is located in granular vesicles (see page 33). Results on the localisation of the amine in other cells, such as blood platelets (Tranzer, Da Prada and Pletscher 1968), enterochromaffin cells (Ito and Winchester 1963), Retzius cells of the leech ganglia (Rude, Coggeshall and van Orden 1969) and the 5-HT-containing cells of the pineal organ (Wartenberg and Baumgarten 1969), all show 5-HT to be localised in the core of granular vesicles. The size of the amine-containing vesicles varies in different tissue. Retzius cells of the leech, cells in the pineal organ, and blood platelets all have 5-HT-containing vesicles which range from 80-120 nm in diameter, whilst the vesicles in enterochromaffin cells are slightly larger, 150-250 nm, in diameter. It definitely appears as if 5-HT is normally localised in granular vesicles, but

unfortunately there does not seem to be a single morphological form of vesicle that can be used to identify the presence of 5-HT.

When 5-HT is localised in cytosomes, the amine is usually associated with the homogeneous parts of the organelles. It is not clear why the amine ever occurs in these organelles, especially since at any one time only some of the cytosomes ever contain 5-HT. These observations may reflect the loss of the amine from some of the particles during the fixation and staining. Alternatively, it may be that the cytosomes pass through several developmental stages as in other situations (Meek and Lane 1964 and Baranyi 1966) and that it is only during certain of these stages that they contain 5-HT. It is of interest to note that Potter and Axelrod (1963) showed NA to be associated with heart microsomes of the rat, and recently van Orden, Vugman, Bensch and Giarman (1967) showed 5-HT to be localised in multivisicular bodies of neoplasmic cells, which also contained two hydrolytic enzymes normally found in lysosomes.

It is possible that granular vesicles are formed in the cytosomes. A definite relationship between the two types of organelles can be recognised. On occasions,

cytosomes are packed with vesicles, whilst at other times granular vesicles appear to be released from the organelles. (see fig 53). A similar relationship between agranular vesicles and 'lysosomes' (cytosomes) in the neuropile regions of Helix pomatia has been noted by Chalazonitis (1968). He obtained photographs of 'lysosomes' containing granular vesicles, and also showed the release of these vesicles from the organelles. However there is a lot of convincing evidence implicating the Golgi complexes in the formation of vesicles (see Bourne 1962). The Golgi complexes of the 5-HT neuron are always associated with granular vesicles.

What is the function of 5-HT within cytosomes? It is possible that the only way that 5-HT exists in cytosomes is in granular vesicles, and that other formations are artefacts. The multivesicular bodies observed by van Orden, Vugman, Bensch and Clarman (1967) could then be synonomous with the cytosomes. It is interesting that Chalazonitis (1968) showed the occurrence of 'lysosomes' which contain either clear or granular vesicles. He suggested that the clear vesicles were really empty granular ones and that their neuroamines had been liberated. The function of the released amine is postulated as exercising a regulating role on the electric properties of neighbouring membranes (Chalazonitis 1968).

CONCLUSIONS

(1) The pair of giant cells located in the metacerebral ganglia of *Limax maximus* contain 5-HT and no other monoamines.

(2) The amine is present in the cytoplasm of the neuron.

(3) 5-HT is localised in small granulated vesicles which range from 60-120 nm in diameter at all times of the year.

(4) In early spring, 5-HT is additionally localised in lysosome-like particles or cytosomes.

(5) The concentration of 5-HT in the neurons is estimated to be 7 ± 1 ng/cell.

DISTRIBUTION OF MONOAMINES IN CONSTRICTED VISCERAL NERVE

INTRODUCTION

The aim of these experiments was to investigate the transport of 5-HT from the central nervous system to the heart. Previous work has shown that the amine is localised in small granulated vesicles and also under certain conditions in lysosome-like particles or cytosomes in neurons of the slug brain (see previous section), whilst in axons of the heart of Helix, 5-HT could be found only in granulated vesicles (see Cottrell and Osborne 1969b).

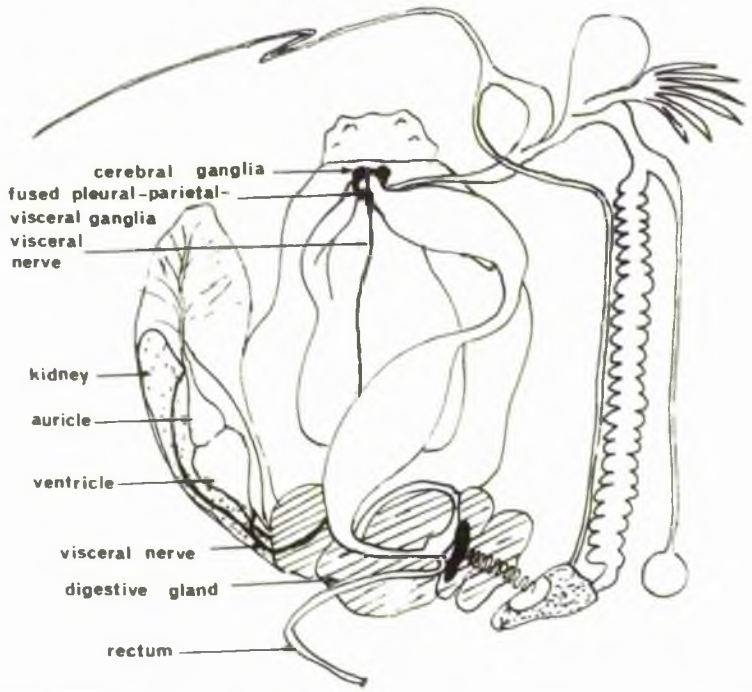
METHODS

Part of the shell was removed from the snail Helix pomatia, and the animal pinned down securely on to a layer of wax at the base of a small dissecting dish. The visceral nerve leaving the visceral ganglion (see fig 61) was exposed by making an incision along the dorsal surface of the animal and the nerve ligatured at one or two points (0.5-1 cm apart). A ligature was made in the following way. A fine glass rod was placed alongside the nerve, and a fine silk thread tied securely around both nerve and rod. After operation, animals were covered with Merg's saline, into which air was continuously bubbled. The saline was changed several times immediately

Fig 61. Dorsal view of the central nervous system and the position of the visceral nerve of Helix.

Fig 62. Amine-specific fluorescence observed in a longitudinal section of the visceral nerve of Helix pomatia. (The bar represents 0.2mm).

61


cerebral ganglia
fused pleural-parietal-visceral ganglia
visceral nerve
kidney
auricle
ventricle
visceral nerve
digestive gland
rectum

62

after the operation and then two to three times a day until the nerve was removed. Operated animals were kept for a maximum of three days before removing the ligatured nerves and either freeze-dried for fluorescence microscopy or fixed in glutaraldehyde for electron microscopy. In other instances the 5-HT concentration in visceral nerve 1 cm on either side of ligatures was estimated by biological assay on the isolated Helix aspersa heart (see page 125).

Glutaraldehyde fixed tissues were subsequently either post-fixed in osmium tetroxide or reacted with dichromate (Wood's method) and then processed for electron-microscopy.

RESULTS

1 Fluorescence in unconstricted nerve

Spread preparation of whole nerve revealed a dull specific fluorescence but it was rarely possible to distinguish individual nerve fibres, because of the thickness of tissue. Sections of nerve tissue showed greater detail. In longitudinal and transverse sections, individual monoamine containing fibres could be followed easily. The fluorescence intensity of fibres depended upon the diameter of nerves at the level of the section. Often bundles of 5-20 fluorescent fibres could be found running

parallel to each other (fig 62). Fluorescent fibres were relatively smooth and showed an even distribution of monoamines. On very rare occasions the fluorescence within fibres appeared banded. The fluorescence was yellow-green in colour and reached a maximum intensity after 1 hour of formaldehyde sublimation at 80°C indicating the presence of 5-HT and DA and/or NA. The visceral nerve had very little natural fluorescence.

2. Effect of constrictions on fluorescent fibres.

Fluorescence indicative of amines was seen on either side of ligatures up to 15 hours after the operation. During the succeeding 20 hours there was a rapid increase in the fluorescent material proximal to the constriction and a decrease in the distal region (fig 63). Visceral nerves ligatures at two points showed an increase in fluorescence proximal to each constriction, but the intensity of fluorescence was greater above the proximal constriction (fig 64). Occasionally, specific fluorescence could be followed into constricted regions, probably because in such preparations the ligatures were not made tight enough to occlude all the individual fibres in the nerve trunk. Transverse sections of regions of the visceral nerve proximal to constrictions showed that the fluorescence was confined within bundles of fibres (fig 64).

Fig 63. The distribution of amine fluorescence in the region of constriction (k) of a length of visceral nerve. Figs a,b and c show three sections of the same preparation. The intensity of fluorescence is high in the region proximal (p) to the constriction but very low in the constricted portion itself and in the region immediately distal (d) to it.
(Each of the bars represent 0.2mm).

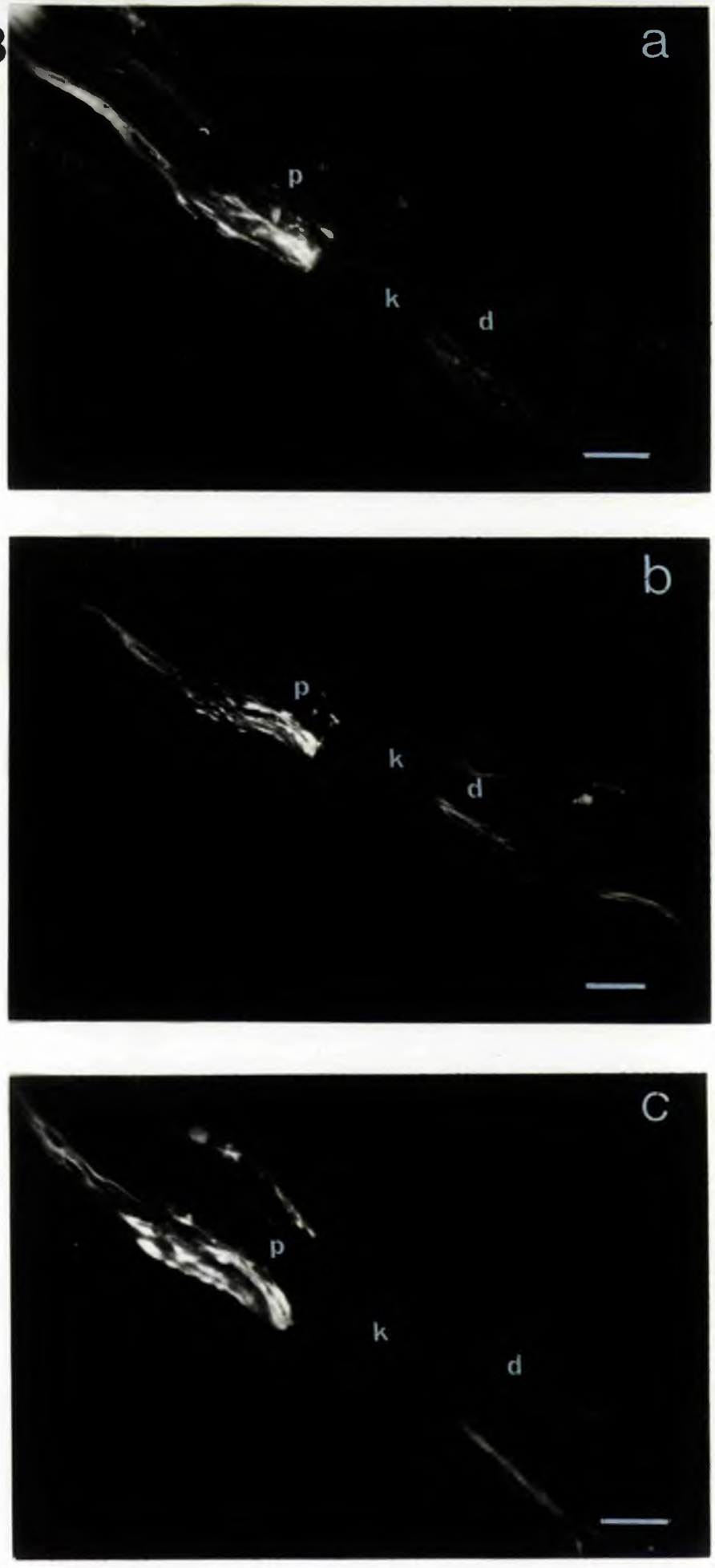
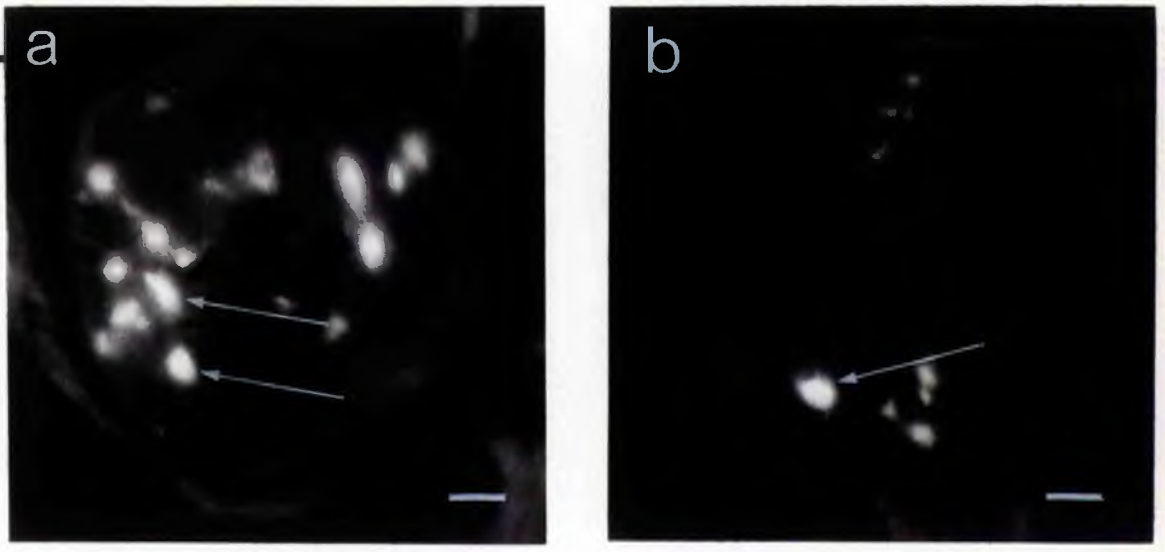

a
p
k
d
b
p
k
d
c
p
k
d

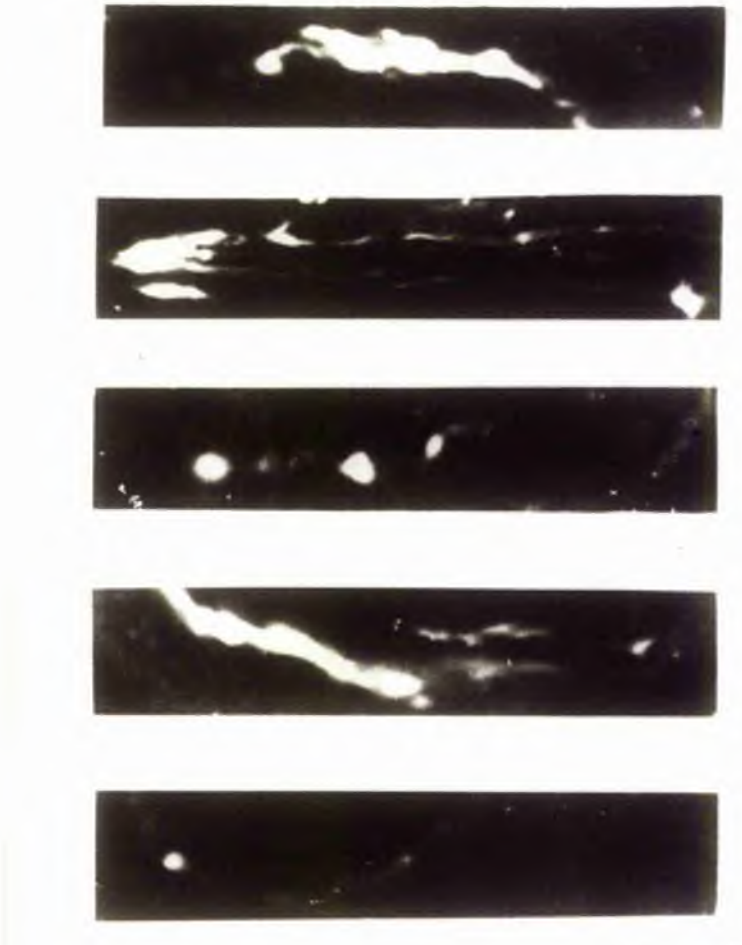
Fig 64. Transverse sections of the proximal regions of visceral nerve constricted at two points (a) The first section of the proximal part of constriction made nearest to the brain and (b) the first section of the proximal part of constriction furthest away from the brain. There is a greater amount of amine-fluorescent (arrows) nerve bundles in (a) than (b).
(Each of the bars represent 0.1mm).

Fig 65. Examples of amine-containing fibres on the proximal side of constrictions. Morphologically, the nerve fibres resemble typical regenerating nerves.
(The bar represents 1μ).

64



65



Fibres proximal to constrictions, exhibited great morphological variations compared with normal smooth fibres (fig 65). Fluorescent intensity in different areas of individual fibres often varied greatly to give structures which resemble regenerating fibres (see Weiss and Hiscoe 1948) such as growth cones, bulb-like enlargements, ballooning and coiling.

3. Electron Microscopy

The visceral nerve is made up of a number of nerve fibres, cell bodies and glial cells. The axons are generally ensheathed and vary from 0.3–3μ in diameter. The nerve fibres contain large numbers of vesicles and mitochondria, occasional formations of endoplasmic reticulum and large tubular mitochondria. Cytosomes, similar to that observed in the giant 5-HT cell were not observed. The most conspicuous structural elements of the axon were constituted by vesicles. They ranged from 30–150 nm in diameter and were either clear (agranular) or possessed a dense core (granular). The description of axons after constriction will be concerned mainly with these vesicles, some of which contain monoamines.

There was a small but definite increase in the number of vesicles compared with normal, in some axons

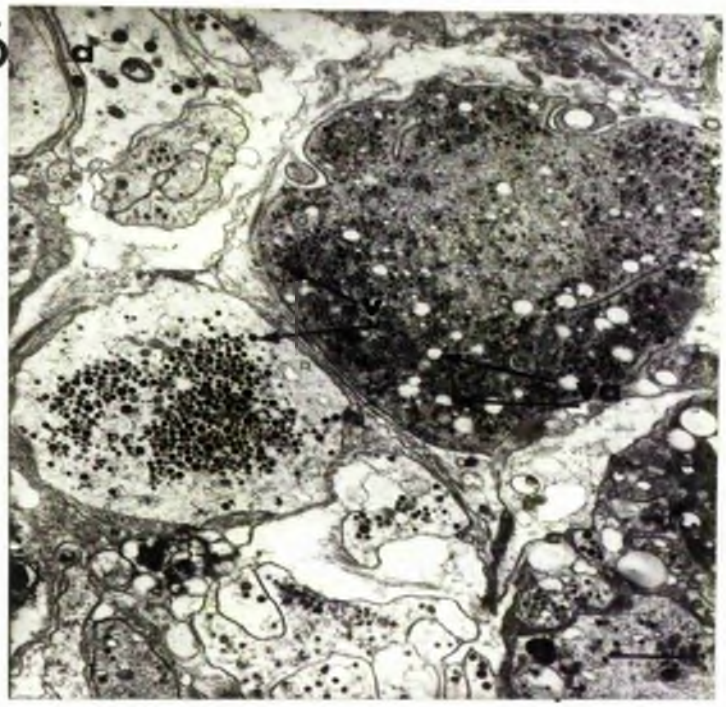
proximal to constrictions as early as 5 hours after operations. Striking changes within fibres were observed 20 hours after constrictions. Axons on that side of the ligatures nearest to the brain became swollen and filled with granular and/or agranular vesicles (figs 66 & 67). Many vacuoles and mitochondria were also present. From 30 hours after operations, the picture was further complicated by the presence of numerous fibres which probably represent regenerating axons. The majority of these fibres also contained vacuoles.

Despite all variations in different axons after operations, most striking were the early onset and rapid accumulation of granular and agranular vesicles proximal to the constrictions. Histochemical studies, based on the chromaffin reaction (Wood's method), provided evidence that amines are associated with some of the granular vesicles. They were the only type of particle to give a positive reactive electron dense deposit (fig 68).

Considerable changes in ultrastructure were seen in the region distal to constrictions, even in preparations which had been ligatured for only 6 hours. The changes are too complex to be described in detail here, and for proper interpretation more experiments of shorter and longer duration

Fig 66. Large numbers of small granular vesicles (V) are observed in sections of nerves prepared from the area of nerve proximal to constriction. In these examples, the nerve had been ligatured 20 hours before preparing for microscopy. Some nerve fibres appeared darker than others, and contained gramular vesicles of diameters ranging from 30-100 nm. Gramular vesicles of about the same size were observed in the lighter axons. Another feature of most axons was the presence of large vacuoles (va). (Each of the bars represent 0.5μ).

66



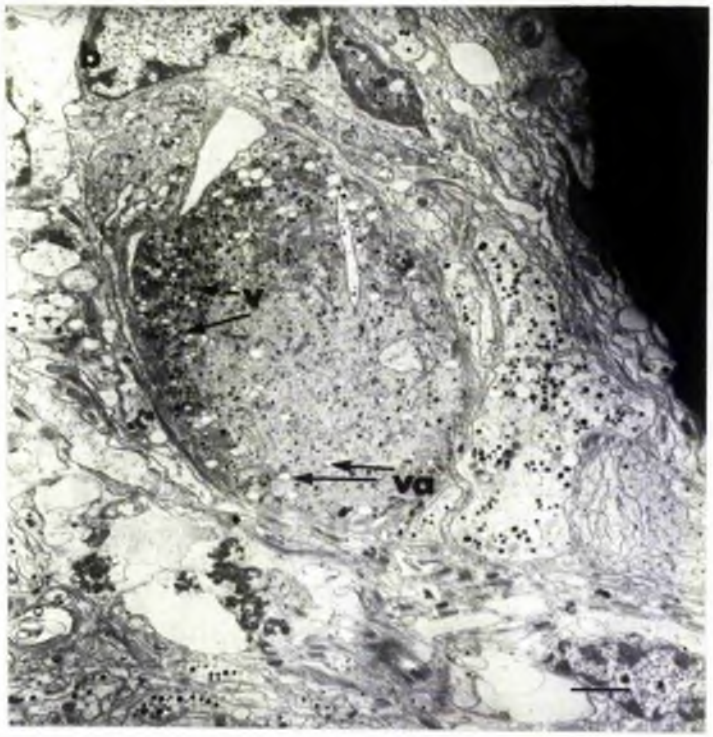
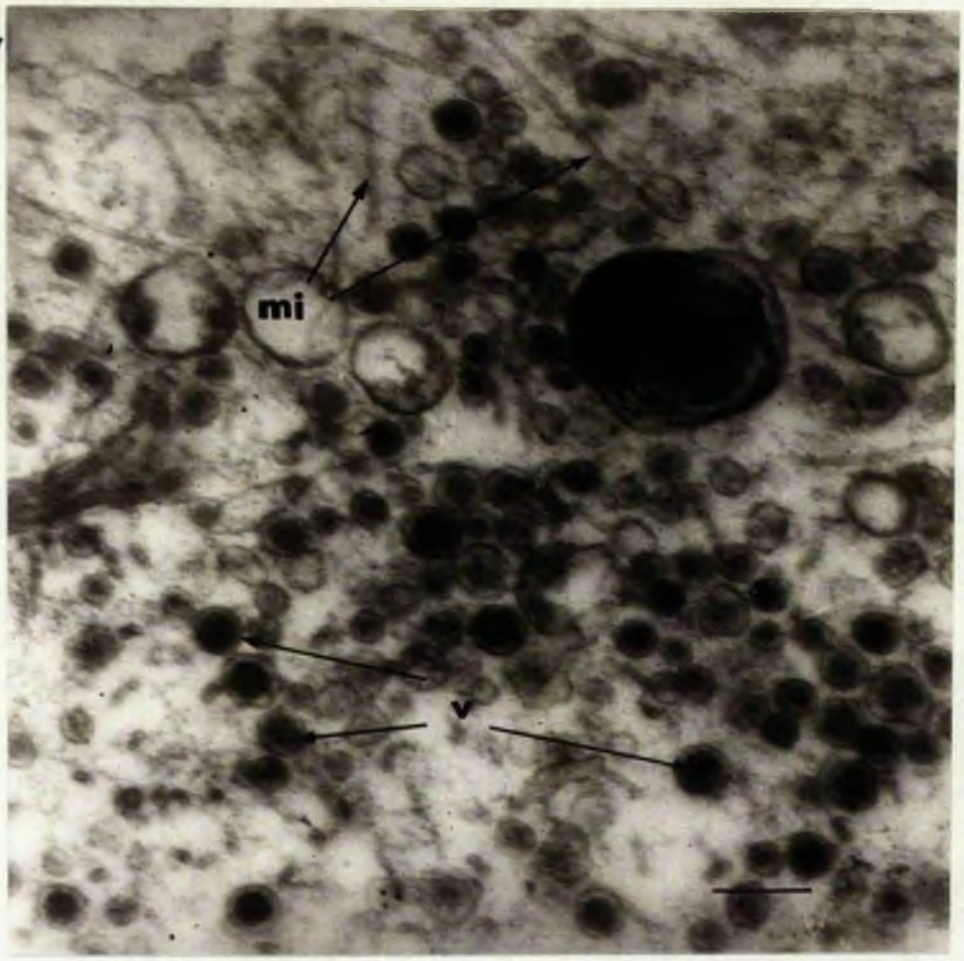


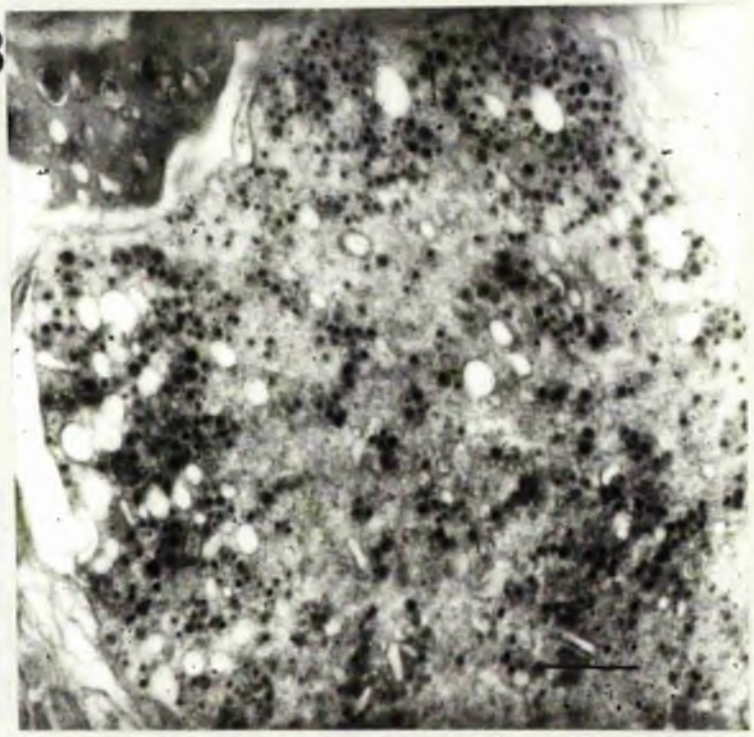
Fig 67. Granular vesicles (see fig 66) at a higher magnification. The electron dense deposits are in the centre of the vesicles (v). Some microtubules (mi) can also be seen.
(The bar represents 0.2μ).

Fig 68. Electron dense material, representing the location of an amine compound, in the small granular vesicles of nerve fibres. The section was prepared from the region of visceral nerve proximal to a constriction. The nerve was ligatured for 20 hours before fixing for electron microscopy.
(The bar represents 0.5μ).

67



68



seem necessary. The axonal contents were disorganised and contained a variety of membranous structures, some granular and agranular vesicles, many vacuoles and swollen mitochondria (fig 69). There was no increase in the number of vesicles, and tissues processed by the Wood's method did not show a large number of electron dense granules, as seen proximal to constrictions.

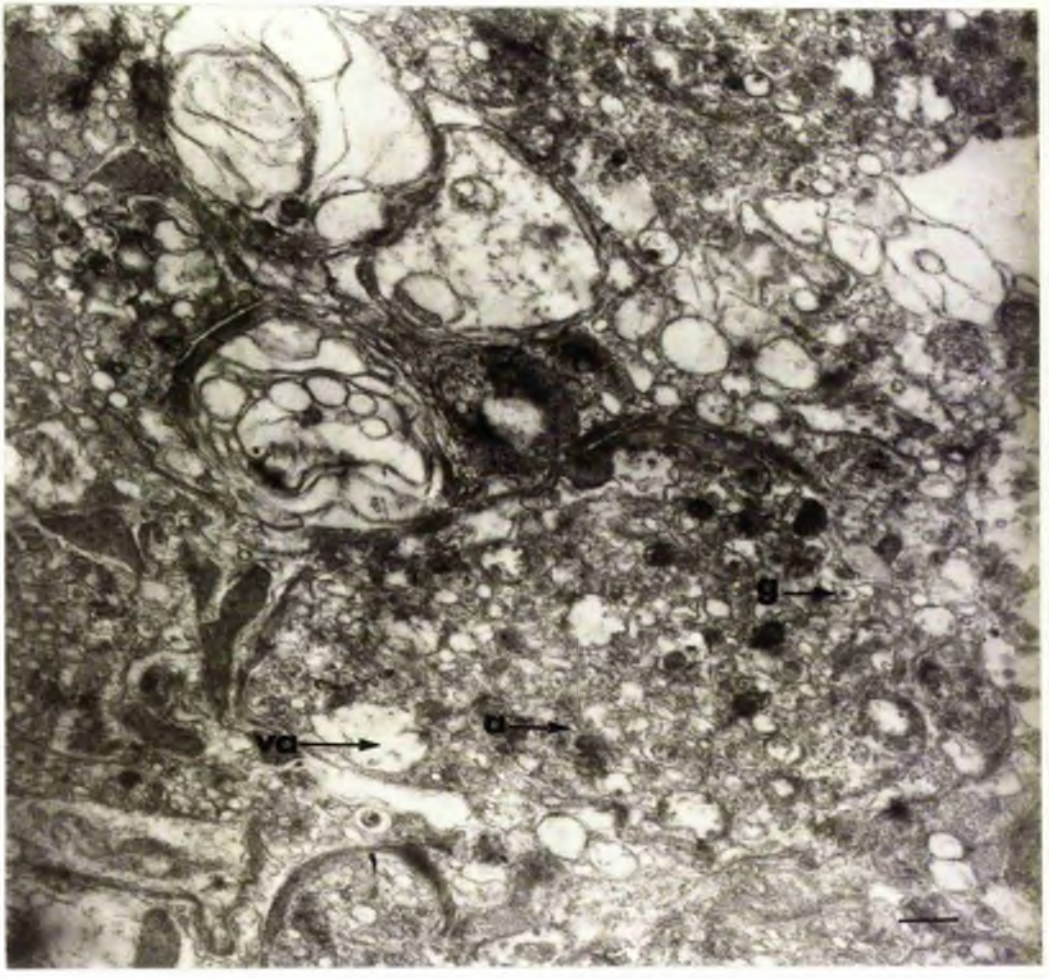
4. Biological Assay of 5-HT.

Extracts of visceral nerve were prepared from 5-10 animals, by homogenising tissue in 0.5-1 ml Ringer's saline. Addition of visceral nerve extracts to the snail heart led to an increase in amplitude and frequency of the heart beat. This response is like that elicited by low concentrations of 5-HT. In fig 70, we see the effect of application of similar amounts of visceral nerve extracts prepared from different portions of the visceral nerves. It is clear that the substance which excites the Helix heart in a similar way to pure 5-HT, accumulates proximally and decreases distally in constricted nerves, as compared with the normal nerve content. It is estimated that the unconstricted visceral nerve contains 70 ng of 5-HT/g, the proximal part of ligatured nerve 200 ng of 5-HT/g and the distal part 20 ng of 5-HT/g.

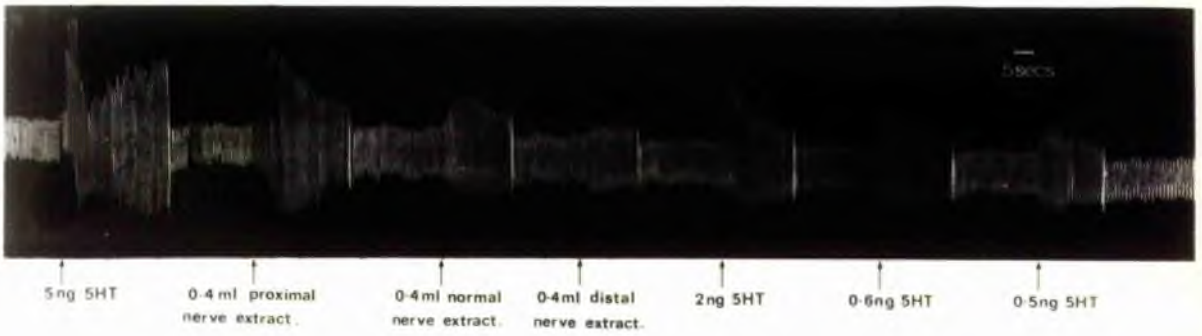
Fig 69. The appearance of an area of nerve trunk distal to a ligature. Enlarged axons contain vacuoles (va), agranular (a) and granular (g) vesicles. Complex arrangements of membranes are common. (The bar represents 0.5μ).

Fig 70. Responses of the isolated Helix aspersa heart to extracts prepared from regions of visceral nerve proximal and distal to constrictions, as well as from non-ligatured nerve, and different concentrations of 5-HT. The different nerve extracts contained the same concentration of tissue. Extracts prepared from the region proximal to the ligature were most potent in causing cardio-excitation.

69


g
vg
g

70


5secs
5ng 5HT
0·4 ml proximal nerve extract.
0·4ml normal nerve extract.
0·4ml distal nerve extract.
2ng 5HT
0·6ng 5HT
0·5ng 5HT

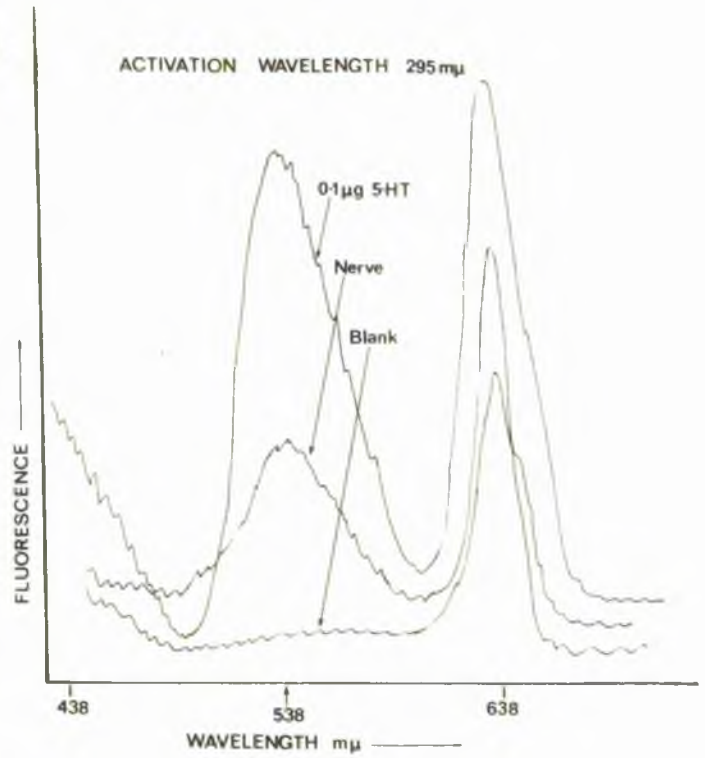
DISCUSSION AND CONCLUSIONS

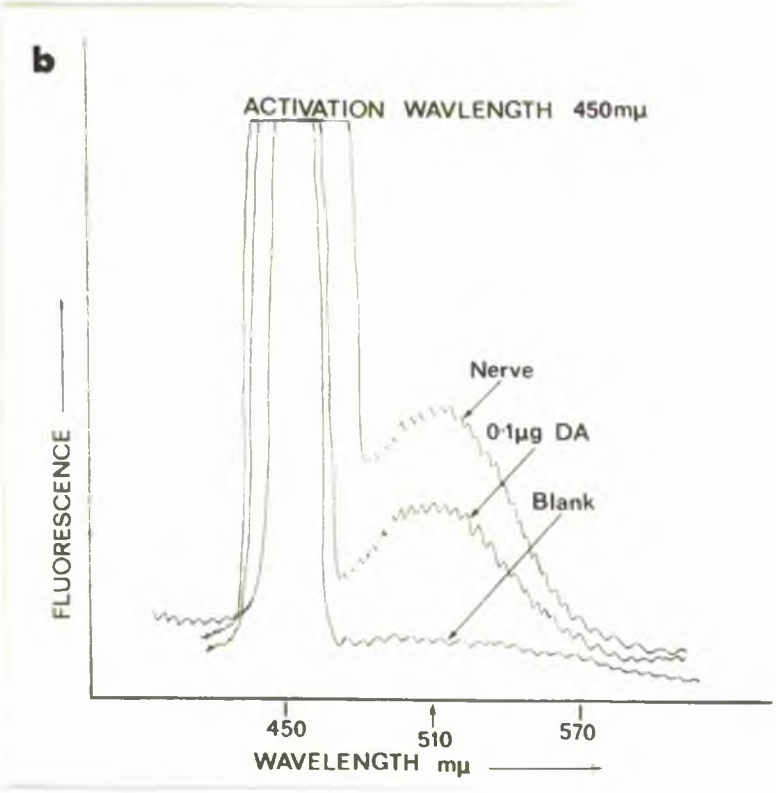
The visceral nerve of the snail was chosen for the present experiments because it is easily recognisable, long enough, contains a relatively moderate amount of monoamine fibres and innervates the heart. Spectrophotofluorometry has shown that primary catecholamines and 5-HT are present in the visceral nerve (see fig 71 for details). For this reason the results obtained are applicable to the monoamines in general; certain conclusions concerning 5-HT transport are however possible.

The rapid accumulation of specific amine-fluorescence above the proximal constriction suggests that the movement of monoamines from the central nervous system to nerve terminals in peripheral tissue had been blocked. The fact that monoamines also accumulate above the distal constriction, but not below the proximal one, at least after the first 15 hours from the operation, suggests that movement is predominantly from the ganglia to the periphery, and that it may to some extent be independent of the effect of the cell bodies from neurons. In some cases fluorescent nerves were also found distal to the constrictions. They were of low intensity compared with the fluorescent materials found proximally to the constrictions. Dahlström (1965) observed a similar and more intense accumulation of fluorescent material on the distal side of constricted adrenergic nerves.

Fig 71. Fluorescence spectra to show the presence of (a) 5-HT and (b) primary catecholamine in the visceral nerve of Helix pomatia. The method used to detect primary catecholamines (Shore and Olin 1958) does not differentiate between DA and NA.

71


a
ACTIVATION WAVELENGTH 295mμ
0·1μg 5-HT
Nerve
Blank
FLUORESCENCE
438
538
638
WAVELENGTH mμ


b
ACTIVATION WAVLENGTH 450mμ
Nerve
0·1μg DA
Blank
FLUORESCENCE
450
510
570
WAVELENGTH mμ

The morphology of fluorescent structures, proximal to constrictions from 30 hours after operations are almost identical to the variety of configurations described by Cajal (1928) and Weiss and Hiscoe (1948) as being associated with regenerating nerves. Similar findings using the fluorescence method have also been reported by Dahlström (1965) Blümcke and Niedorf (1965) and Olson (1969) in constricted nerves of vertebrates.

The biological assay experiments showed that at least part of the monoamine accumulation proximal to ligatures is due to 5-HT. The level of 5-HT estimated on the proximal side of ligatures was greater than in that of similar amounts of non-constricted nerves, whereas the level in distal regions was lower than both. These results provide extra evidence for a peripheral flow of 5-HT along the visceral nerve.

Estable, Acosta-Ferreira and Scotelo (1957) were the first to show accumulation of vesicular components 20-70 nm proximal to the stump of a sectioned sciatic nerve from the guinea pig. Recently, the accumulation of vesicles in nerves has been studied in conjunction with the histochemical fluorescence method. Small granular vesicles were also found by Kapeller and Mayor (1967) in compressed splenic and hypogastric nerves, and by Dahlström and Haggendal (1966) in unmyelinated axons of sciatic nerve.

In both cases the finding of granular vesicles was correlated with an increase in fluorescence of catecholamines.

The present experiments confirm previous observations in the vertebrates. A large accumulation of vesicular material at the proximal stump of ligatured visceral nerve is observed. The vesicles are both granular and agranular in appearance. That the granular vesicles contain monoamines is confirmed in tissue cytochemically stained by the Wood's method. The Wood positive granules correlate with the high intensity of fluorescence proximal to constrictions. In none of the electron micrographs studied were lysosome-like particles or cytosomes observed.

These experiments also show that movement of the monoamines in the visceral nerve is predominantly unidirectional, namely from cell bodies in the central nervous system to their nerve terminals. Such results have been postulated by Breeman, Anderson and Reger (1958) who believe that synaptic vesicles originate in the perikarya, and by Weiss, Taylor and Pillai (1962) who considered changes in the proximal stump of a constricted nerve a manifestation of axonal flow. Geffen and Ostberg (1969) have recently provided evidence which shows NA-containing granular vesicles to be synthesised in the cell body and then transported to the axon terminals in sympathetic neurons.

The occurrence of a small but significant amount of monoamines distal to constrictions can be explained in the following ways: (a) by the presence of ganglion cells distal to the constrictions (b) by both way streaming of axonal contents (c) by inadequate constrictions of nerves. The last possibility was excluded by cutting the nerve. The idea of bi-directional flow of axoplasm has been formulated by Lubinska (1964) and applied by Dahlstrom and Haggendal (1966) and Kapeller and Mayor (1967) to explain the axonal flow in compressed nerves. If this is applicable to present results, one would expect to find fluorescence on all occasions associated with distal parts of constrictions, instead of on rare occasions. In contrast, cell bodies have been observed in many parts of the visceral nerve, by use of both fluorescence and electron microscopy. It was however impossible to relate them with the ultra-thin fluorescent axons distal to constrictions.

From these experiments, it is concluded that monoamines located in small granular vesicles are transported from cell bodies in the central nervous system towards peripheral nerve terminals of the visceral nerve. The question of retrograde flow of monoamines in either normal

or constricted axons must remain open. The results further show that whereas 5-HT is associated under certain circumstances with cytosomes in nerve perikarya (see page 140), the amine is not associated with such particles in axons of the visceral nerve and therefore not transported in this form.

GENERAL DISCUSSION

During the past decade there has accumulated considerable evidence which indicates that monoamines act as neurotransmitters in a wide variety of species. This contention is supported by the high concentration within certain neurons of monoamines and the enzymes which synthesise and inactivate them, and also by their storage within synaptic vesicles, their high rate of turnover in nervous tissue and the effects of their microelectrophoretic application upon spontaneous and stimulated neuronal activity. Furthermore, biochemical and fluorescent histochemical evidence for the release of neuronal monoamines has been reported (see Introduction and Review of Literature). The most important features in chemical transmission are the nature of the transmitter substance, and the post synaptic membrane. There are several criteria which define a transmitter substance (see Salmoiraghi, Costa and Bloom 1965; Gerschenfeld 1966; and Werman 1966). The following are the most important:

1. The presumed transmitter should act on the post synaptic cell exactly as in nervous transmission. The substance should affect the synaptic membrane, and the

time course of its action should be comparable to that of the synaptic potential. Also, the changes of the ionic conductance of the membrane should be the same during synaptic transmission as in the presence of the applied substance.

2. The presumed transmitter should be present in the pre-synaptic terminal and bound in such a form (i.e. synaptic vesicles) that a certain number of transmitter molecules are released to correspond with quantal units.

3. Enzyme systems present for its synthesis should be located at the synapse.

4. Drugs applied to the synapse should affect synaptic transmission in a similar way to the action of applied transmitter substance.

5. After presynaptic stimulation, there should be a release of the substance from the terminals in concentrations sufficient for nervous transmission.

There are only a few instances of synaptic transmission in which the transmitter can be identified according to all these stringent criteria. For example, this is true of cholinergic transmission in the vertebrate neuromuscular junction, of adrenergic transmission in visceral muscle, and of GABA in the crustacean neuromuscular junction. Because of the great technical difficulties

involved, the last criterion is often impossible to satisfy and so we must not exclude the possibility of a suspected transmitter substance just because it does not fulfil all five points. Each substance should be judged on its own merit. The results presented in this thesis support previous results in providing strong evidence for monoamine neurotransmission in the gastropods.

Thin layer chromatography and spectrophotofluorometry have shown the occurrence of DA and NA metabolites in the whole circumoesophageal ganglionic mass and heart of Helix aspersa. The presence of NA metabolites was especially interesting for it was previously thought that NA was absent from tissue of gastropods. It was also of interest that 5-hydroxyindoleacetic acid, the major metabolite of 5-HT could not be detected. The significance of this is not clear, especially since 5-hydroxyindoleacetic acid is found in high concentrations in parts of vertebrate tissues known to contain 5-HT. Yet trace amounts of 5-hydroxytryptophol, a minor metabolite of 5-HT in the vertebrates, were found in the circumoesophageal ganglia mass. Except for Kerkut's and Cottrell's survey (1963) of a number of snail tissues (heart, brain, kidney and blood) for MAO activity, and their

discovery that only the kidney can oxidise 5-HT to 5-hydroxy-indoleacetic acid, there was previously almost nothing known about the breakdown of monoamines in the gastropods. On the other hand, there are a lot of data concerning the formation of monoamines in the gastropods. The synthesis of catecholamines may be considered to begin with the hydroxylation of tyrosine to form DOPA (see fig 1). Tyrosine (Kerkut and Cottrell 1962) and DOPA (Sedden, personal communication) have been detected in the blood of Helix aspersa. No work has yet been carried out on the occurrence of the enzymes tyrosine hydroxylase and dopamine-β-hydroxylase or on their sites of action. Fluorescence microscope studies have suggested that DOPA decarboxylase is present in the cell membrane of neurons. Injection of DOPA causes a green fluorescence greater than that normally seen in catecholamine neurons. Pre-treatment of snails with DOPA decarboxylase inhibitors prevents the green fluorescence which could mean that DOPA is decarboxylated in the cell membrane and so passed to the perikaryon. Similar studies also suggest that 5-HTP decarboxylase is present in cell membranes of the neuron. Work by Kerkut and Cottrell (1963) and Cardot (1963) has proved the existence of 5-HTP decarboxylase in the nervous system of snails. However, attempts to show the existence

of 5-HTP have failed, presumably because the substance is unstable or perhaps because it has a very high turnover.

Until now, attempts to detect NA in gastropod molluscs have been unsuccessful (Sweeney 1963; Kerkut, Sedden and Walker 1968) but in the course of the present studies a combination of bioassay and chromatography has revealed its occurrence in snails. In chromatographed Helix heart extracts, DA and 5-HT are also present, though the function of primary catecholamines in the gastropod heart is not clear. Fluorescence microscopy shows the presence of both DA and NA in some of the heart nerves. Furthermore the primary catecholamines are effective in altering the mechanical activity of the isolated heart, but only, in high concentrations compared with 5-HT, the presumed excitatory transmitter (see page 115). Rózsa and Ks-Nagy (1967) and Rózsa (1969) have proposed that cardio-excitation of the snail heart is through a chain-like sequence and that one of the steps involves the catecholamines. This hypothesis is based on results which concluded that 5-HT is localised in muscle cells and not nervous tissue.

Paper chromatography and spectrophotofluorometry were used to estimate the 5-HT content in the thick walled muscular ventricle and in the fragile auricle parts of

Helix heart. Results show that the auricle (0.29 µg/g 5-HT) has about twice the amine concentration of the ventricle (0.15 µg/g 5-HT). This argues for a specialised localisation of 5-HT rather than a uniform distribution in muscle cells, which is in disagreement with the view of Rózsa and Zs-Nagy (1967). They proposed that the amine was present in muscle cells, after being unable to find 5-HT-containing nerves in the heart of Lymnea stagnalis.

Conventional histology and electron microscopy revealed the distribution of nerves in Helix heart as sparse throughout the musculature, and dense in the auricular side of the auricular-ventricular junction. In view of electron microscopy cytochemistry by Gottrell (see Gottrell and Osborne 1969a and Gottrell and Osborne 1969b) it was decided that the profuse network of nerves in the auricular-ventricular junction is neurosecretory in function and that some of the other neurons contain monoamines. Application of fluorescence microscopy together with the selective use of drugs which interefere with the metabolism of 5-HT, revealed that the amine is present in cardiac nerves. These results have recently been confirmed by Chase, Reese, Carpenter and Koplin (1968), and Taxi and Gautron (1969), who all showed that some of the cardiac nerves of Aplysia contain 5-HT by a variety of methods: fluorescence microscopy, radio-autography, electron microscopy and electrical

stimulation to release labelled 5-HT from the heart. These results are important, for although there was a strong case for 5-HT acting as the normal cardio-excitatory transmitter in the gastropod heart, the amine had not previously been shown to occur in the cardiac nerves.

The presence of 5-HT and DA in the whole circumoesophageal mass of Helix pomatia has been confirmed by paper chromatography and spectrophotometry, and the level of each amine corresponds to that found in other studies (see page 75). Biochemical, pharmacological and physiological data on the occurrence of DA and 5-HT in molluscs, show that they are probably synaptic transmitter substances in the central nervous systems of gastropods. (for reviews see Florey 1965; Gerschenfeld 1965 and Cottrell and Laverack 1968). The most important data implicating DA as a synaptic transmitter are summarised by Ascher 1968; Walker, Woodruff, Glaizner, Sedden and Kerkut (1968), and Kerkut, Horn and Walker (1969). The work of Gerschenfeld and Stefani (1968) on the CILDA neurons of snails, has contributed to provide one of the best examples of 5-HT acting as a chemical transmitter in the animal kingdom.

There is a low concentration of NA in the brain, but the low level does not necessarily directly reflect a proportionately less important functional role than compared with DA. It is possible that the low level of NA may represent a higher rate of turnover than that of DA. However the relatively small quantities of NA breakdown products in the brain suggest that the rate of NA turnover cannot be very much greater than DA. Most likely both catecholamines act as transmitters in the central nervous system (page 53). The observation of Claisner (1967) that certain neurons in the ganglia of Helix aspersa respond to NA but not to DA agrees with the view that NA serves such a role.

Fluorescence microscopy was used to study the cellular localisation of monoamines in a number of tissues of the slug, in order to obtain information regarding their functions in peripheral tissues. Results indicate that nerves containing 5-HT are often associated with muscle tissue. It has been shown that 5-HT relaxes anterior byssus retractor muscle of Mytilus (Twarog 1954) and penis retractor muscle (Jaeger 1967), radula retractor muscle (Fänge and Mattisson 1958) and pharyngeal retractor muscle of gastropods (Kerkut and Cottrell 1965). It thus

appears that 5-HT is primarily involved in either relaxing musculature or regulating excitatory tissue such as the heart. Myhrberg (1966) has suggested that yellow fluorescence caused by 5-HT in the musculature of annelids is associated with motor functions.

Fluorescence indicative of primary catecholamines is associated with many sensory systems such as the retina of the eye and the statocyst. Many neurons containing catecholamines were located on all parts of the integument; they were especially abundant on the tentacles and anterior body regions. Their widespread distribution and localisation, suggest that they are sensory in function and that different groups of cells respond to a variety of stimuli. Similar bipolar catecholamine cells have been said to occur in the epithelium of sea anemones (Dahl, Falck, Mecklenburg and Myhrberg 1963b) and the earthworm (Rude 1966; Myhrberg 1967).

Dahl, Falck, Lindqvist and Mecklenburg (1962) were the first to show that some of the giant neurons in the brain of gastropods contain either 5-HT or primary catecholamines. During the present programme of work a survey of monoamine fluorescence in the central nervous system of Limax maximus revealed the presence of a single identifiable giant neuron in each of the metacerebral ganglia. These cells were shown to contain a single

monoamine, 5-HT. Isolated cells were assayed on the Helix aspersa heart preparation which is known to be very sensitive to low concentrations of 5-HT. From the results of a number of experiments the 5-HT content of a single cell was estimated to be $\pm$ .1 ng. This value is of the same order as that calculated by Rude, Coggeshall and van Orden (1969) for the 5-HT content in individual cell bodies in the ganglia of the leech Hirudo medicinalis.

Knowledge of the subcellular localisation of 5-HT in nervous tissue is important for the understanding of the precise physiological role of this amine in the gastropods. Attempts have been made to establish the true subcellular localisation of 5-HT in ganglia of molluscs (Zs-Nagy, Zóna, Salanki, Feldes, Peronyi and Demeter 1965; Cottrell 1966 and Cottrell and Maser 1967). In all instances results were inconclusive because tissues examined contained other monoamines besides 5-HT. The giant neurons present in the metacerebral ganglia of the slug were studied critically for the localisation of the amine at the electron microscope level. Results show that 5-HT is localised in two types of organelles in the cytoplasm of the cell. Ganglia removed from slugs during the summer months showed the amine to be present in a number of small granules. Tissue studied earlier in the year indicated that 5-HT was additionally localised

in a number of lysosome-like particles or "cytosomes", (Nolte, Breucker and Kuhlmann 1965).

The localisation of 5-HT in granules is consistent with other work. Literature on the subcellular localisation of monoamines in molluscs (see page 33) infers that 5-HT is located in a similar type of granule. Results of the distribution of amines in individual cells such as platelets (Etcheverrey & Zieher 1968, Tranzer Da Prada & Pletscher 1968), enterochromaffin cells (Ito and Winchester 1963), Retzius cells of the leech (Rude, Coggeshall and van Orden 1969), and the pineal organ (Wartenberg and Baumgarten 1969) all proved that 5-HT is present in cytoplasmic granules, although the size of the granules varies in different tissues. The precise function of the amine when localised in cytosomes is not clear. There may be a relationship between the occurrence of 5-HT in the two types of organelles as discussed on page 138. However, the presence of "synaptic-type" vesicles provides evidence for implicating the amine as a transmitter substance.

The giant 5-HT neurons in the cerebral ganglia of Limax maximus are also present in the other pulmonate gastropods examined (see page 117). Kandel and Tauc (1966a,b) previously studied the membrane properties of these neurons in Helix aspersa and Helix pomatia and

discovered that they show a high degree of rectification. They also showed, using electrophysiological methods, that each cell send an axon to the ipsilateral exterior lip nerve and one to each cerebral buccal connective. Cottrell (1969) has recently studied these cells in Helix pomatia. and has been able to show that the two giant 5-HT cells form excitatory links with other neurons in the buccal ganglia. The excitatory response of the "buccal cells" also occurred when pure 5-HT (10^{-7} g/ml) was applied to them. Furthermore, response of the cells in the buccal ganglia to activation of the 5-HT neurons was blocked with a relatively low concentration of lysergic acid diethylamide (10^{-5} g/ml) and Methysergide (10^{-4} g/ml). Cottrell also showed that reserpine antagonised transmission between the giant 5-HT cells and "buccal cells". This is to be expected if 5-HT is used as a transmitter substance by the giant cells, for I have shown that reserpine depletes the amine from these cells. The results of this work and the known subcellular localisation of 5-HT in these giant neurons, provide very strong evidence that 5-HT acts as a neurotransmitter in this situation.

The experiments which show the distribution of monoamines in the constricted visceral nerves of Helix pomatia were primarily carried out to investigate the transport of 5-HT from the central nervous system towards the heart. It was also hoped to elucidate the importance of

the role of 5-HT in cytosomes. Fluorescence microscopy and electron microscopy of the ligatured visceral nerve showed that monoamines move from the ganglia towards the periphery. Biological assay of 5-HT content on either side of the ligature showed that there is an accumulation of 5-HT proximal to the ligature. Moreover, fine structural studies of the nerve revealed an accumulation of a large number of vesicles with dense core centres on the proximal side of the ligature. That these vesicles contain monoamines was confirmed by tissue cytochemically stained by a modified chromaffin reaction (Wood, 1966,1967). The nature of the granules is similar to that observed in the giant 5-HT neuron in the metacerebral ganglion of the slug. However no evidence was obtained for the accumulation of cytosomes on either side of the ligatured nerves. These results show that 5-HT and primary catecholamines are present in granules of a similar appearance, and that at least a proportion of the granules is produced in the soma and then transported to their terminals. Moreover, inability to discover an accumulation of cytosomes on the proximal side of the ligature raise speculations concerning the function of 5-HT when present in these organelles.

There is therefore a considerable amount of evidence for monoamine neurotransmission in the heart and

central nervous system of gastropod molluscs. For convincing proof, a preparation would have to be found in the circumesophageal ganglia where an identified monoamine neuron could be stimulated, the substance collected, identified and tested for its physiological action on the post junctional neuron. Ascher, Glowinski, Tauc and Taxi (1968) have attempted to demonstrate that exogenous tritiated 5-HT and DA can be accumulated by nervous structures of Helix and Aplysia, and can be liberated from these structures by electrical or chemical stimulation. Their initial results were good but later autoradiography pictures of incubated ganglia, together with biochemical data on ganglia sheaths and other non nervous structures, showed that exogenous monoamines were taken up and released from the connective tissue sheath instead of from nervous tissue.

Although all the available data indicate that 5-HT is the cardio-excitatory substance in gastropod heart, the mode of action is not known. It is possible that 5-HT activates certain biochemical processes in the muscle cells as does AD in the vertebrates (see Sutherland and Robison 1966; Robison, Butcher and Sutherland 1968). Mansour, Sutherland, Rall and Bueding (1960) have shown that adenylcyclase of the invertebrate Fasciola (liver

fluke) can be activated by 5-HT. Furthermore the work of Mansour (1954, 1967) has provided evidence that 5-HT can accelerate anaerobic glycolysis by effects, which are probably mediated by cyclic 3'5' monophosphate (C-AMP) on phosphorylase and phosphofructokinase. Adenyl cyclase is present in the heart of Spisula solida and the enzyme is stimulated with 5-HT (Cottrell and Osborne 1969b). It seems therefore possible that C-AMP may be involved in response of the heart of 5-HT. However, the best way of resolving the problem concerning the mode of action of 5-HT would be to record intracellularly from individual cells of the heart. In this way one could test the effect of 5-HT and other substances directly on heart cells.

The significance of 5-HT fluorescence in other muscle tissues of the gastropods still has to be established. The amine might participate in similar processes as have been suggested in heart tissue. Moreover, 5-HT has several effects on Mytilus smooth muscle: a relaxation of catch, increase in spiking, decrease in membrane resistance and stimulation of metabolism (see Twarog 1968). It seems, therefore, that the presence of 5-HT in each type of muscle tissue has to be judged independently and we should not exclude the possibility that the amine functions as a neurotransmitter agent in one instance and more as a hormone in another.

Experimental proof is needed to determine the significance of catecholamine fluorescence outside the central nervous system. The green fluorescence in sensory structures is of special interest. First, it has to be established whether the fluorescence represents one or both of the primary catecholamines. Again, the fluorescence in the neuropile regions of the digitate ganglion is very interesting, for this structure presumably participates in the sensory activities of the optic tentacles. This system may be suitable for studying the true subcellular localisation of DA and/or NA.

SUMMARY

An outline of our present knowledge concerning the occurrence, distribution and possible function of monoamines in the animal kingdom has been reviewed. The purpose of the present study was to investigate the localisation of monoamines in gastropod molluscs so as to gain some insight into their functional significance. The main aim was to find out whether the monoamines, particularly 5-HT, function as neurotransmitter agents.

The following observations were made:

1. By a combination of spectrophotofluorometry and thin layer chromatography the DA metabolites 3,4-dihydroxyphenylacetic acid, homovanillic acid and the corresponding metabolites of NA 3,4-dihydroxymandelic acid and vallinmandelic acid were detected in brain and heart extracts of *Helix*. Greater quantities of the NA products were present in the heart than in the brain, whereas the brain contained the higher level of DA metabolites. Thin layer chromatography also showed the presence of small quantities of the 5-HT metabolite, 5-hydroxytryptophol in each of the extracts. There was no evidence for the occurrence of 5-hydroxyindoleacetic acid, p-hydroxyphenylacetic acid and p-hydroxymandelic acid, metabolites of 5-HT, tyramine and octopamine respectively in either heart or brain extracts of *Helix*.

2. Chemical and biological assay methods confirmed the occurrence of DA (3-6 µg/g of brain tissue and 0.4-0.8 µg/g of heart tissue) and 5-HT (2-4 µg/g of brain tissue and 0.2-0.4 µg/g of heart tissue) in Helix tissue. In addition it was established that the brain contains 0.8 µg, and the heart 1 µg of NA per g fresh weight of tissue. The fact that there is a greater concentration of NA in the brain than the heart whereas in the case of DA the reverse is true corresponds with a similar distribution of each amine's metabolites.

3. The intrinsic innervation of the Helix heart show a sparse distribution of axons in all areas of the heart and a dense network of nerves in the auricle side of the auricular-ventricular junction. The dense network is positioned close to, or bordering on, the heart cavity. The nerves have pronounced swellings along their lengths and are filled with electron opaque granules similar to these seen in known neurosecretory systems. On the basis of this morphological data, and also because the nerves do not contain monoamines it is thought that the network serves a neurosecretory function.

4. Amine-fluorescence histochemistry shows monoamines to be localised in the "sparse nerve fibre system" which is seen throughout the Helix heart. Further evidence for the presence of 5-HT as well as DA and NA in some of these nerve fibres

was obtained by observing the effects of different drugs on their amine-fluorescence. Results from spectrophotofluorometry, which showed the auricle (0.29 µg/g) to contain greater amounts of 5-HT than the ventricle (0.15 µg/g), together with the fact that amine-fluorescence was only seen in nerve fibres, suggest that 5-HT does not occur in muscle cells.

5. Monoamine-containing neurons are localised in a number of peripheral tissues (heart, integument, tentacles, penis retractor muscle, sole of foot, kidney, alimentary canal, reproductive organs and tentacular, pharyngeal and cephalic retractor muscles) and in the central nervous system of Limax maximus. Catecholamine neurons were mostly associated with sensory structures such as the statocysts the retina of the eye and the integument of the tentacles. Nerve cells containing 5-HT were primarily located in muscle tissues. The amount of 5-HT containing, and catecholamine-containing perikarya in the central nervous system is small when compared with the non-fluorescent perikarya. All the ganglia except the proto-cerebral, have some amine-containing neurons, although relatively large numbers of fluorescent cells were observed in the cerebral, visceral, pedal and right parietal ganglia.

6. By amine-fluorescence histochemistry a giant 5-HT containing neuron was observed in each of the meta-cerebral

ganglia of Limax. Further evidence for 5-HT and the absence of catecholamines in the giant cells was obtained by observing the effects of different drugs on the fluorescence. Furthermore, biological assay of cell extracts provided independent evidence for the presence of 5-HT in the neurons, and the amount of amine was estimated to be 7 ± 1 ng/cell. A number of organelles e.g. granular vesicles (40-120 nm in diameter), cytosomes, Golgi fields, mitochondria and a few multivesicular bodies, all possible candidates for 5-HT localisation, were observed throughout the cytoplasm. Results from electron microscopic cytochemistry showed that the amine is sequested in the small granular vesicles and is also localised in the cytosomes at certain times of the year.

7. Visceral nerve extracts of Helix pomatia are shown to contain 5-HT and primary catecholamines. Constriction of the nerve resulted in a marked increase in the level of the amine-fluorescence proximal to the ligature and a reduction in the level distal to the ligature. Concomitant with the increase in fluorescence, there was an increase in the number of small granular vesicles (30-150 nm in diameter) in the nerve fibres proximal to constrictions. It was estimated by biological assay that the unconstricted visceral nerve contains 70 ng of 5-HT/g, the proximal part of ligatured nerve 200 ng of 5-HT/g and the distal part 20 ng of 5-HT/g.

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